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ELECTRON MICROSCOPIC STUDIES OF ADENOSINETRIPHOSPHATASE ACTIVITY IN BLOOD VESSELS AND ITS RELEVANCE TO VASCULAR PERMEABILITY

V. T. MARCHESI

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ELECTRON MICROSCOPIC STUDIES OF ADENOSINETRIPHOSPHATASE ACTIVITY IN BLOOD VESSELS AND ITS RELEVANCE TO VASCULAR PERMEABILITY

V.T. Marchesi

Department of Anatomy
Yale University School of Medicine

Presented to Yale University
in partial fulfillment
of the requirements
for the degree of
Doctor of Medicine

1963

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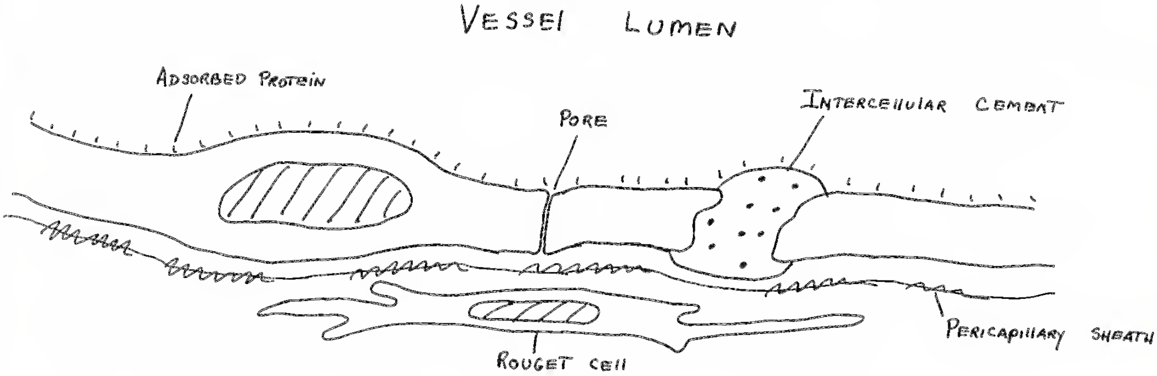
INTRODUCTION

Speculation on the mechanism by which materials are transported across the endothelium of blood vessels has often been based on a hypothetical conception of the vessel wall since, previous to studies with the electron microscope, the detailed structure of small blood vessels was not known. Some of these conceptions were derived from results of perfusion studies of isolated vascular beds (1-4). As a result of such studies the structure of the blood capillary was hypothesized to be as depicted in fig. 1.

Chambers and Zwiefach considered that an intercellular cement substance, thought to be demonstrated by silver staining and carbon perfusion studies (4), was the site through which materials passed from the blood stream into the tissues. They further postulated that changes in the permeability of vessels were the result of changes in the consistency and porosity of this cement substance.

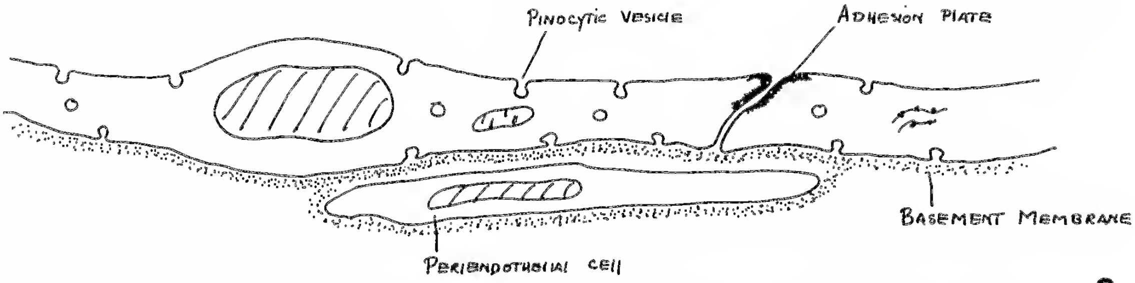
Pappenheimer and coworkers (2, 3) perfused isolated hindlimb preparations of animals, and, on the basis of the calculated rates of disappearance of substances in the perfusate, hypothesized that materials passed across the vessel wall through small (30-45Å) pores. They speculated that the pores might extend either through the endothelial cells or through the intercellular cement substance.


VESSEL LUMEN
ADSORBED PROTEIN
PORE
INTERCELLULAR CEMENT
PERICAPILLARY SHEATH
ROUGET CELL

1

Studies of the structure of small blood vessels with the electron microscope have provided more precise information on the structure of the vessel wall. Fig. 2 represents a model of the electron microscopic version of the same type of vessel depicted in fig. 1. The wall of the vessel was found to be made up of endothelial cells joined together by intercellular junctions; the cells were closely applied and no gaps or pores were seen between them. There was no evidence of any substantial intercellular cement substance between the cells, nor was there any indication that a layer of protein was adsorbed on the luminal surfaces of the endothelial cells. It was conceivable, however, that these substances were either lost in the preparation of the tissues for electron microscopy or that they were not visible in the electron microscope. No pores or channels have been found in the endothelium which might correspond to the pores hypothesized by Pappenheimer (3).

In addition to the usual organelles (nuclei, mitochondria, endoplasmic reticulum, Golgi, and small granular components) found in other cells, the endothelial cells of heart and muscle capillaries were found to contain numerous small vesicles. These vesicles, which measured approximately 600-800Å in diameter were found in greater numbers along the endothelial cell membranes which border both the vessel lumen and the periendothelial spaces. Palade (5) was


Pinocytic Vesicle
Adhesion Plate
Basement Membrane
Periendothelial cell

2

the first to describe the existence of these submicroscopic vesicles, and he suggested that they might serve as a carrier mechanism for the transport of materials and water across the endothelial cells.

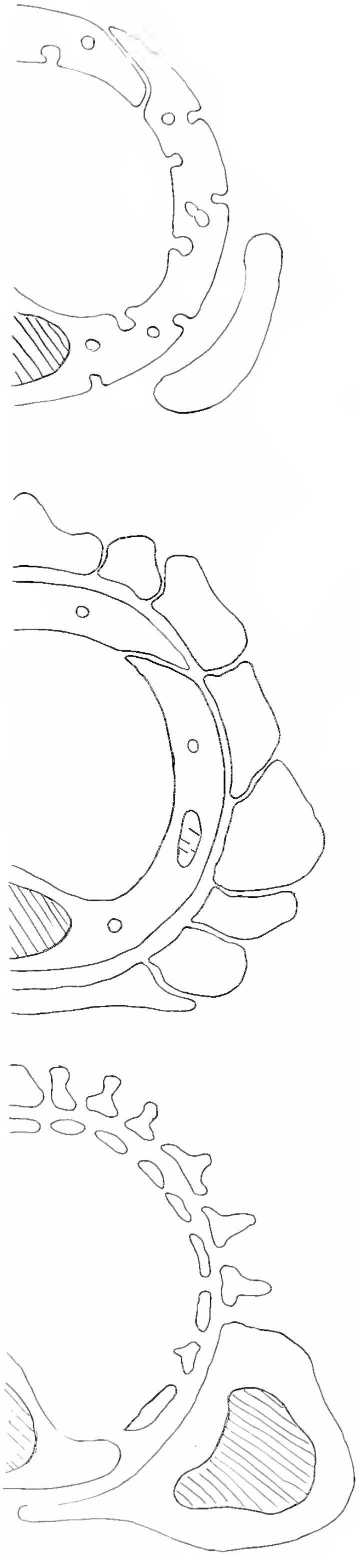
As a result of further studies with the electron microscope it has been shown that the structure of the endothelial cells which line the vessels varies in different tissues (6). At least three "types" of blood vessels have now been described, and they are presented diagramatically in fig. 3. and outlined below.

I. Capillaries of heart (5, 7, 8), skeletal muscle (5, 6), lung (9), and connective tissue (10) are lined by a continuous layer of endothelium which is surrounded by a continuous basement membrane. These vessels are also usually invested by a discontinuous layer of periendothelial cells, and connective tissue. The cytoplasmic vesicles described above are prominent in these vessels.

II. Capillaries of central nervous system (11) and retina (12) are lined by a continuous layer of endothelium which is surrounded by a basement membrane. Directly outside the basement membrane glial cells and their processes form another continuous sheath, and there is no apparent extracellular space. Vesicles are not prominent in these vessels.

III. Capillaries in kidney (glomerular and peritubular) (13-15), intestine (6, 16, 17), and endocrine glands (18-21) are lined by

endothelial cells which contain intracellular fenestrations or discontinuities in their cytoplasm. The endothelial cells are surrounded by a continuous basement membrane. Cytoplasmic vesicles are not prominent in these vessels.

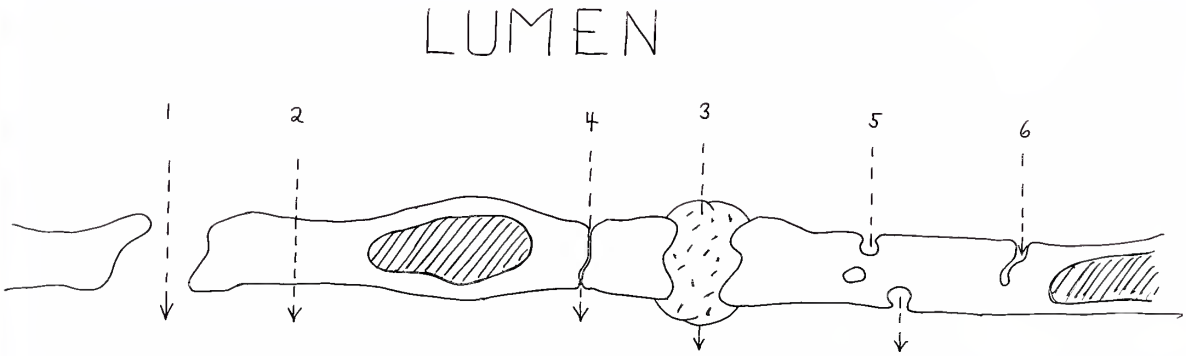


3

Theories of Vascular Permeability

It has been suggested that materials passed across the walls of small blood vessels by one of the following routes:

1. through open intercellular gaps (22)
2. across the endothelial cytoplasm (23)
3. through intercellular cement (1)
4. through pores (3)
5. within pinocytic vesicles (5)
6. by membrane flow (24)



4

Evidence is support of 1-4 was acquired through perfusion studies and analysis of histological preparations with the light microscope. Since it has been shown that the structure of the vessel wall is more complex than could be visualized by the light microscope, several of these hypotheses are untenable if the newer observations are the correct ones. Intercellular gaps have not been seen between the endothelial cells of normal vessels of types I-III (5, 6, 8), nor was there an extensive intercellular cement substance (25, 26). Electron microscopists also reported that no pores of the type hypothesized by Pappenheimer were seen in the endothelium (5, 6).

The hypothesis that materials are transported across the endothelium by way of the cytoplasmic vesicles has attracted many supporters (6, 8, 10, 27, 28). It was suggested that these vesicles functioned by pinching in or engulfing fluid from the blood stream in a manner analogous to the process of pinocytosis described by Lewis (29). The vesicles in the endothelial cells (and those in other cell types as well) have been called pinocytic vesicles, although it appeared that the principal function of these microvesicles was to transport materials across the endothelial cells rather than for incorporation of substances with the cells.

Experimental Studies on the Transport of Substances Across Vascular Endothelium

Attempts to demonstrate experimentally how materials pass across the vessel wall have included studies of normal blood vessels and studies of blood vessels subjected to conditions of acute inflammation. The latter studies were based on the assumption that the mechanism of increased vascular permeability in inflammation involved an accentuation of the normal mechanisms of blood-tissue exchange. However the results of a number of such studies indicated that gaps or openings developed between the endothelial cells in inflammation which allowed materials to pass out of the blood into the tissues (30-34). Since such gaps have not been seen in non-inflamed blood vessels it can be concluded that either they were missed by many observers -which is unlikely- or that the mechanism of increased permeability in inflammation is different from the mechanism of blood vessel-tissue interchange under physiologic conditions.

Studies of the passage of particulate tracer substances (eg. ferritin, iron oxide) across the endothelium of normal vessels have been reported (8, 28, 35), and the results suggested that materials could be transported within the pinocytic vesicles. These findings were not conclusive, however, since the observations related primarily to the uptake of particles by the pinocytic vesicles of the endothelium rather than to their transport across the endothelium.

This problem was investigated further with the perfused heart preparation, and under the conditions of perfusion, particles of iron oxide passed across the endothelial cells of heart capillaries by way of the pinocytic vesicles (36). The electron micrographs which demonstrated this point are presented in figs. 5-9. The results of these studies thus indicated that the pinocytic vesicles in capillaries of type I could function in the transport of substances across the vessel wall.

The permeability properties of the type II vessels in the central nervous system and retina have been the subject of considerable speculation. However little is known of the basis for the selective permeability of these vessels, nor is it clear by what route materials pass across the endothelium and its continuous investment of glial tissues.

Studies on the permeability of type III vessels, the glomerular capillaries, by Farquhar, Wissig, and Palade (37, 38) have demonstrated that the fenestrated glomerular endothelium was not a barrier to the passage of tracer particles. Their evidence indicated that the basement membrane and possibly the foot processes of the epithelial cells were the structures which regulated the degree of permeability.

Figs. 5-9 are electron micrographs of blood capillaries in rat hearts which were perfused with iron oxide particles for varying periods and fixed in osmium tetroxide. These micrographs demonstrate the route the particles take in their passage across the endothelium. These pictures are taken from a study by Drs. Jennings and Florey and the author (36).

Fig. 5. Capillary from a perfused heart. Dense particles are present both in the lumen of the blood vessel (L) and out in the extravascular tissue spaces. An intercellular junction (J) is present in the endothelium (E), and it appears to be intact.

x30, 000

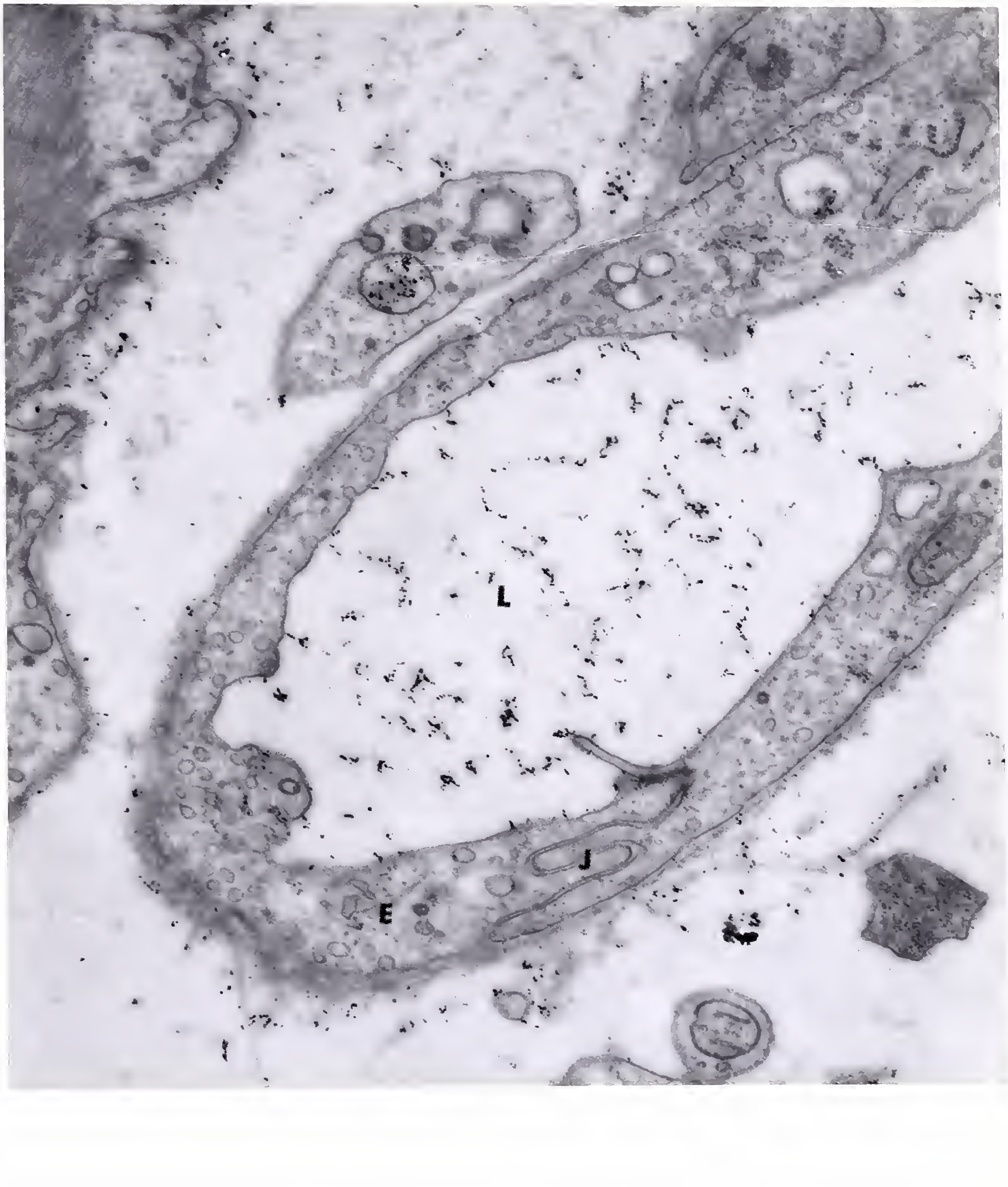

L
E
J

Fig. 6. Capillary from a perfused heart. Particles of iron oxide (arrows) are within vesicles which open on the luminal border of the endothelial cell (E). An intercellular junction (J) is present and intact.

x80, 000

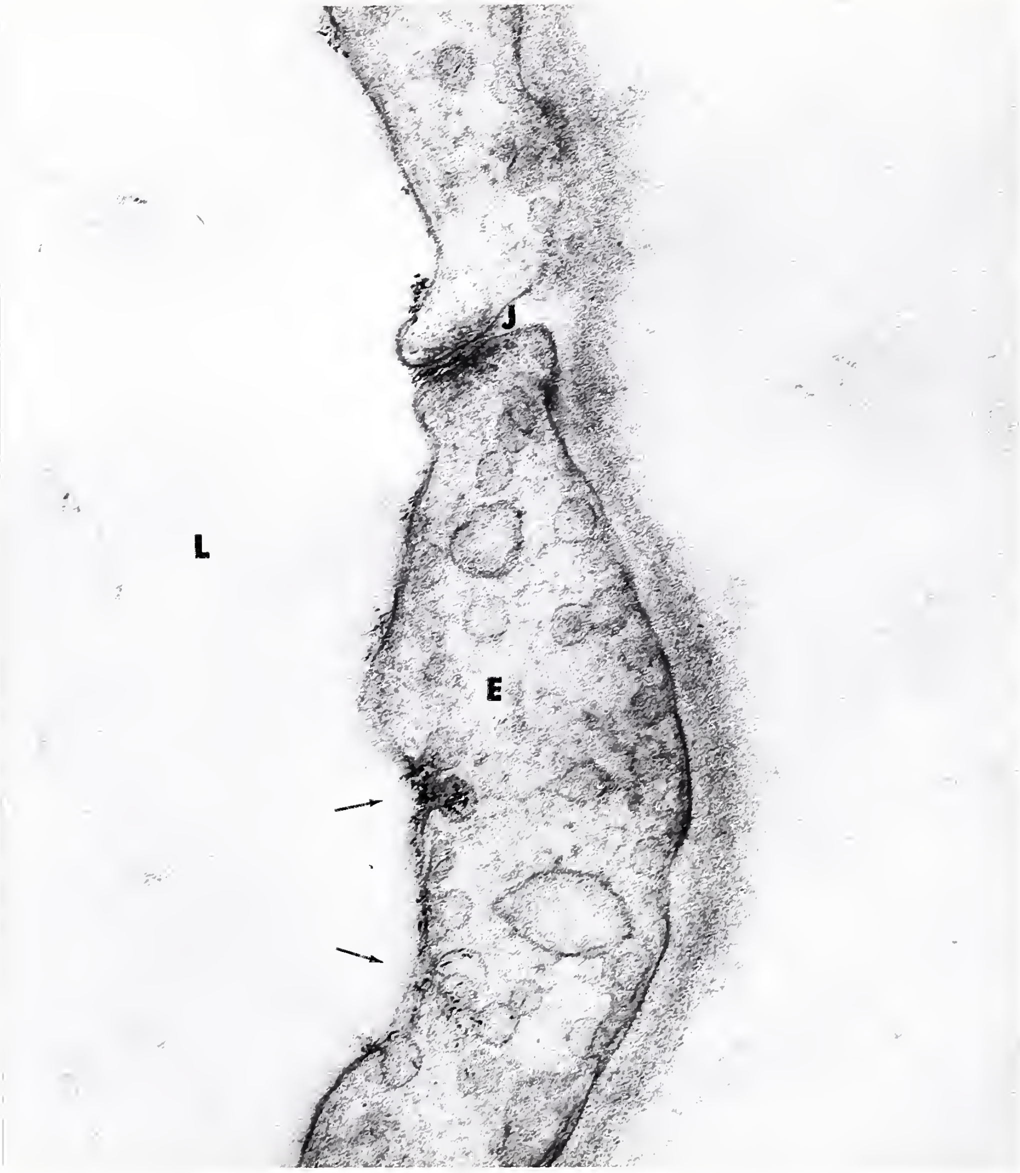

J
L
E

Fig. 7. Capillary from a perfused heart. Particles of iron oxide are present within many vesicles on the luminal border of the endothelium and in some vesicles within the cytoplasm. Particles are not seen in the spaces between the endothelial cells.

x70,000

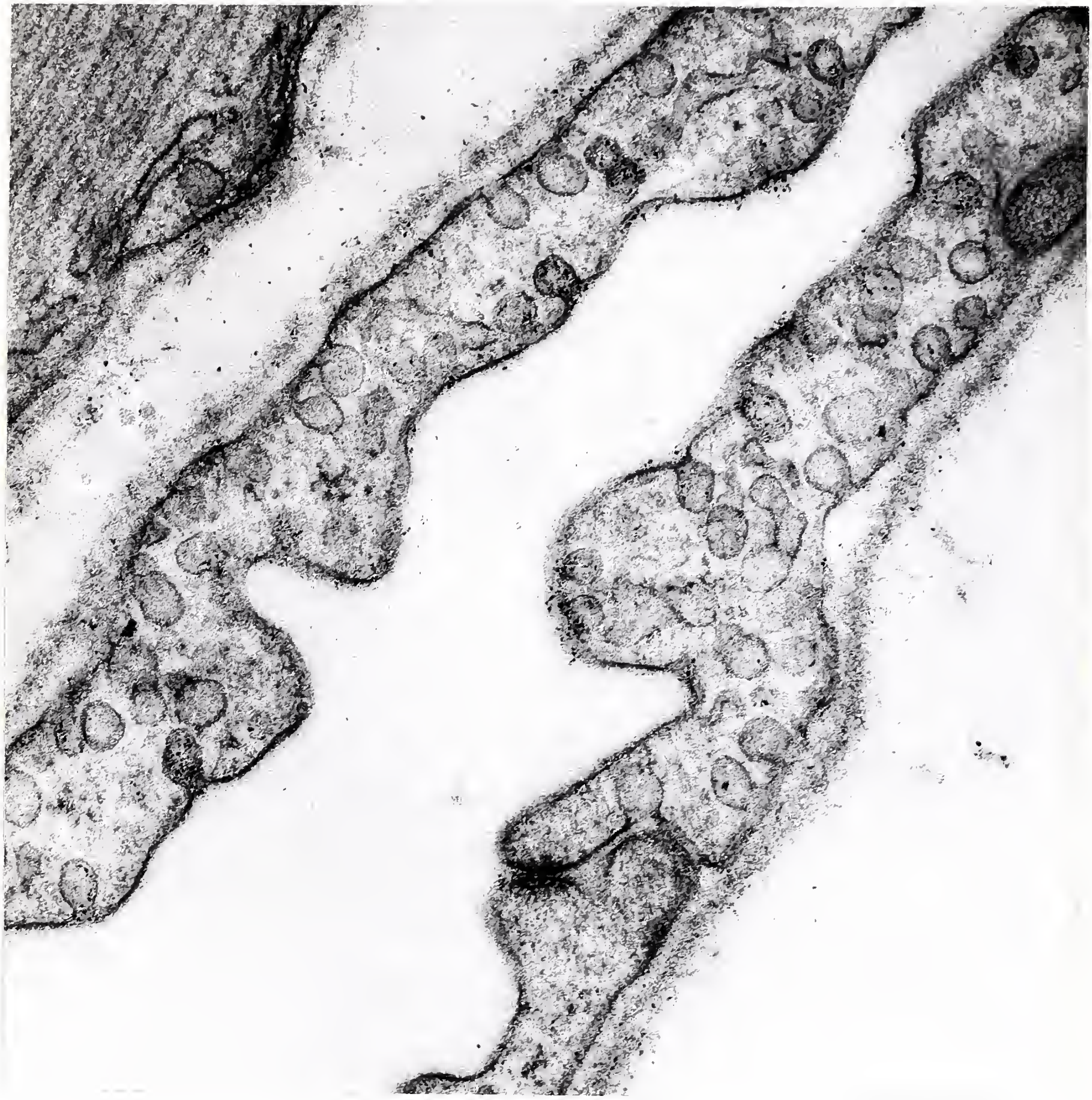


Fig. 8. Capillary from a perfused heart. Particles are seen in vesicles open to the vessel lumen (L), in vesicles open to other vesicles, and a few particles are in vesicles open to the extravacular space.

x125,000

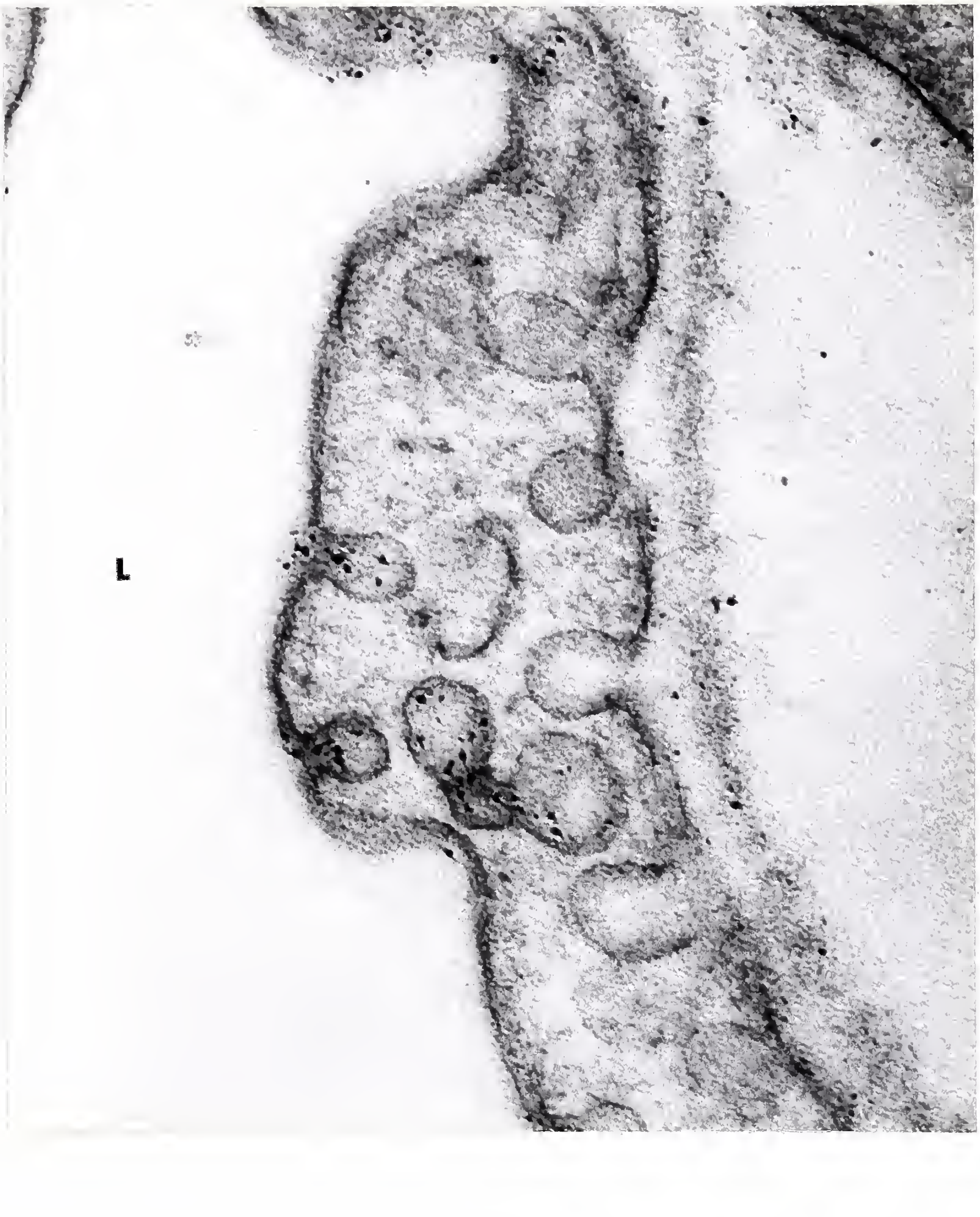
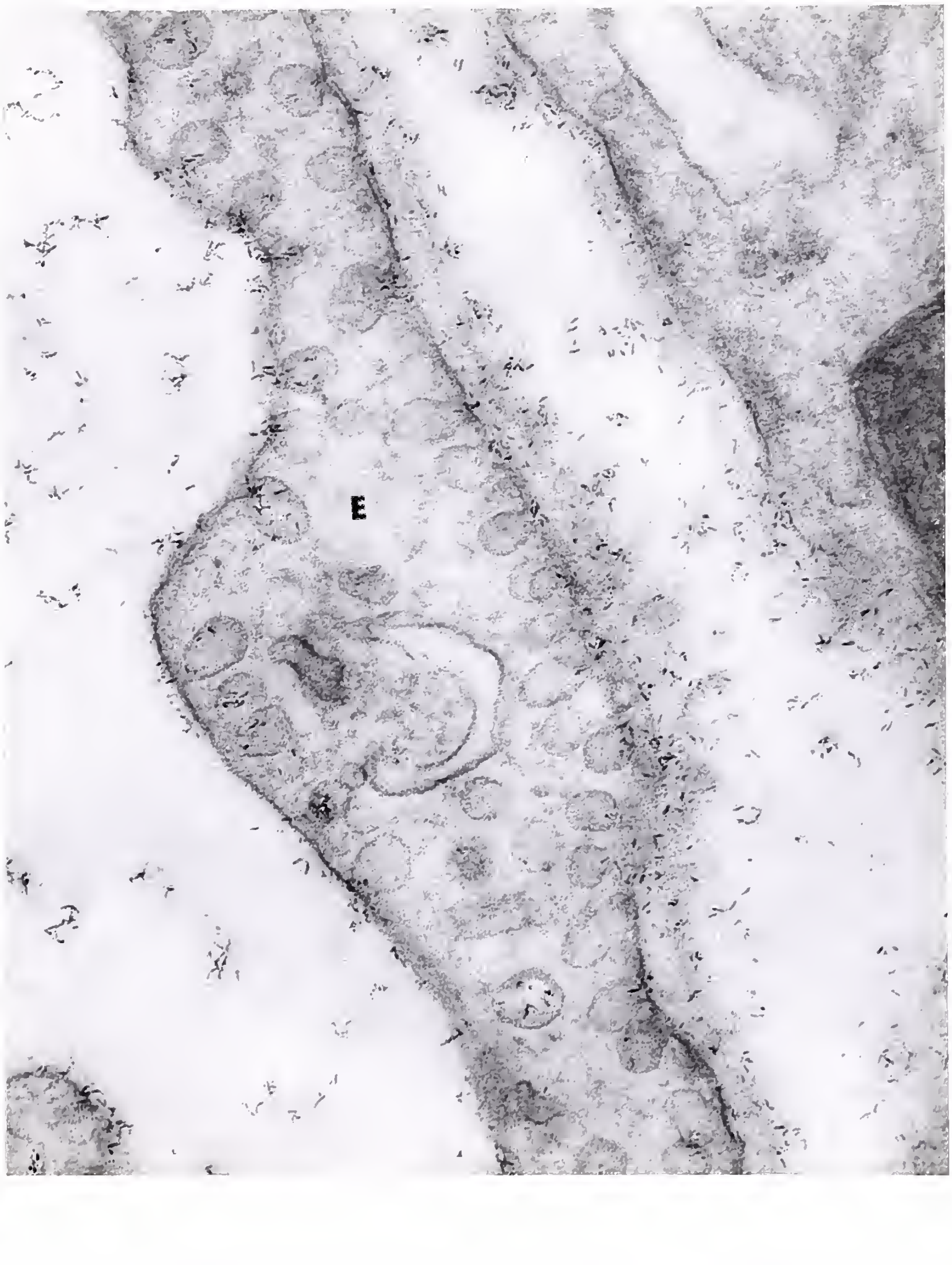

L

Fig. 9. Capillary from a perfused heart. Many particles are seen passing into and out of vesicles which border both the luminal and extravascular surfaces of the endothelial cell (E).

Since no particles have been seen either free in the endothelial cytoplasm or in intercellular spaces, it is concluded that particles pass across the endothelial cytoplasm by way of the vesicles under the conditions of perfusion.

X70, 000


E

Adenosinetriphosphatase Activity and Biological Transport

Studies of a variety of different biological systems have produced evidence which suggested a relationship between the utilization of adenosinetriphosphate (ATP) and ion transport across cell membranes. The early work of Skou (39) indicated that an enzyme (or enzymes) was present in peripheral nerve tissue of crabs which was activated by magnesium (Mg), sodium (Na), and potassium (K), and specific for ATP. By convention this enzyme (s) and those of other tissues which show similar properties have been termed adenosinetriphosphatase (ATPase) activity.

It was shown experimentally that the activity of this ATPase could be correlated with the rates of movement of Na and K across isolated cell membranes. It was demonstrated further that the inhibition of the enzymatic activity by Ouabain resulted in a corresponding inhibition of the Na and K movements (40-44). The most extensive studies of this process have been carried out on human red blood cell membranes, but many other tissues have been found to have similar activity. (45).

It was assumed that the enzymatic activity was probably located on some cell membranes and this localization has been confirmed by the biochemical analysis of isolated cell membrane

fractions (46-48) and histochemical studies of tissue sections (49-52).

Enzymatic activity which utilizes ATP has also been localized to the walls of small blood vessels with histochemical light microscopic techniques (49, 51, 53-55).

The Aim of the Present Study

In the present study an attempt has been made to localize the sites of ATPase activity in the cells of small blood vessels with the electron microscope. Since ATPase activity has been implicated in membrane permeability as described above, it was thought possible that it might also be involved in the process of vascular permeability.

Studies have been carried out on tissues which contain blood vessels of each of the three main types:

1. continuous endothelium with pinocytic vesicles
2. continuous endothelium with a continuous glial investment
3. endothelium with intracellular fenestrations

The sites in blood vessels which showed ATPase activity are demonstrated by representative electron micrographs, and the relevance of these findings to the regulation of vascular permeability will be discussed.

Methods

Freshly excised rat heart, lung, retina, kidney, and small intestine were cut into small pieces and fixed in one of the following fixatives:

6.5% glutaraldehyde (59) buffered to pH 7.4 with 0.1M cacodylate,

12% hydroxyadipaldehyde (59) similarly buffered,

4% formalin buffered to pH7.5 with 0.06M phosphate and containing 7.5% sucrose (58),

1% buffered osmium tetroxide containing sucrose (74). Fixation was carried out in the cold for varying periods of time. Two hour fixation gave adequate preservation of structure and enzymatic activity for glutaraldehyde and hydroxyadipaldehyde and 10 hour fixation was used for formalin. Osmium tetroxide was used as a control fixative which destroyed enzymatic activity in 10 minutes.

After fixation the aldehyde fixed tissues were washed and stored in cold 0.05M Tris or cacodylate buffer, pH 7.4, containing sucrose for periods ranging from overnight to several days. After storage the material was incubated either intact or more finely diced in the standard Wachstein-Meisel adenosinetriphosphatase (ATPase) medium (60) at pH 7.1-7.2, containing lead nitrate as the capture

reagent, either at room temperature or at 10° C. for periods of 15 to 90 minutes. No significant change in pH was noted at the completion of the incubation. Thin (15u) and thick (50u) frozen sections of the same fixed material were incubated along with blocks of tissue. At various times thin sections were removed from the incubating media, treated with ammonium sulphide to develop a visible precipitate (lead sulphide) from the final reaction product, lead phosphate, and examined with the light microscope. When a satisfactory reaction was present in the thin sections, the thicker frozen sections and small blocks of tissue were removed from the incubating media, washed briefly in buffer, and fixed for 2 hrs. in cold buffered osmium tetroxide containing sucrose (74). Tissue processed for electron microscopy was not treated with ammonium sulphide since the final reaction product, lead phosphate, is electron dense.

The same procedures were followed in other experiments in which the ATP in the incubating media was substituted for by beta- glycerophosphate (BG) at equimolar concentrations, and the incubation carried out under the same conditions at pH 7.1. Control experiments consisted of osmium tetroxide fixed tissues (fixed for 10 minutes) incubated in complete medium with ATP and aldehyde fixed tissue incubated in media from which only the substrate was lacking.

After post-incubation fixation in osmium tetroxide the thick frozen sections and small blocks of tissue were dehydrated in a graded series of ethanol and embedded in either Epon (75) or Maraglas (76). Thin sections, cut on an LKB microtome and mounted on Formvar coated grids, were examined without any additional staining in an RCA EMU 3F electron microscope.

Results

Blood Vessels Lined by Continuous Endothelium with Pinocytic Vesicles

Blood vessels of heart and lung were studied as examples of type I vessels. These vessels were found to have vigorous ATPase activity as demonstrated by light microscopy (fig. 11). Control studies included incubation of frozen sections of tissue in medium containing lead but without substrate or in medium containing BG in place of ATP. In both cases no enzymatic activity was apparent (fig. 10).

Fig. 12 is an electron micrograph of a control preparation and is representative of the appearance of vessels in heart fixed in glutaraldehyde, incubated in lead containing medium without substrate, and refixed in osmium tetroxide. The fine structure of the tissue is similar to that found in tissue fixed directly in osmium tetroxide. Numerous pinocytic vesicles are present in the cytoplasm of the endothelial cell. Some of the vesicles are directly continuous with or abutting on the plasma membranes of both the luminal and extravascular surfaces of the cells. The vesicles within the cytoplasm measure between 500 to 800 Å and occur singly or occasionally aggregated. In the latter case either two vesicles are joined together or many vesicles form a rosette pattern. There is no evidence of any non-specific lead precipitate in any of the control preparations.

Fig. 10. Control Preparation Light photomicrograph of a frozen section of myocardium incubated with beta glycerophosphate (BG) and buffered at pH. 7.1, The blood vessels are unreactive under these conditions.

Fig. 11. ATP Reaction. Light photomicrograph of a frozen section of myocardium incubated with adenosine triphosphate (ATP) and buffered at pH 7.1. Dense reaction product outlines the small blood vessels.

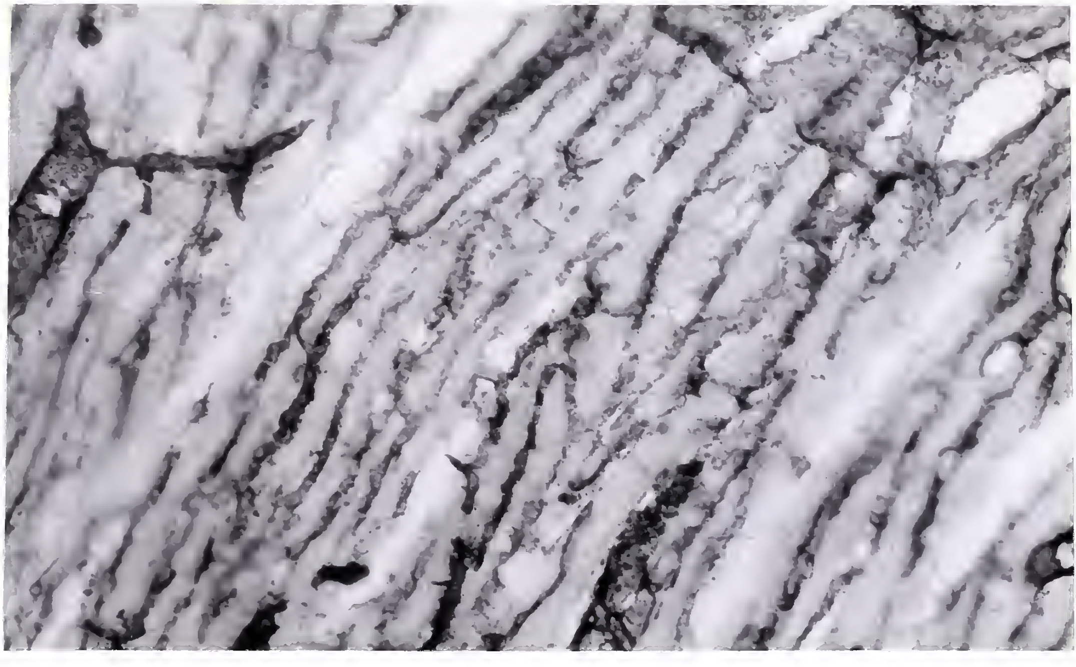
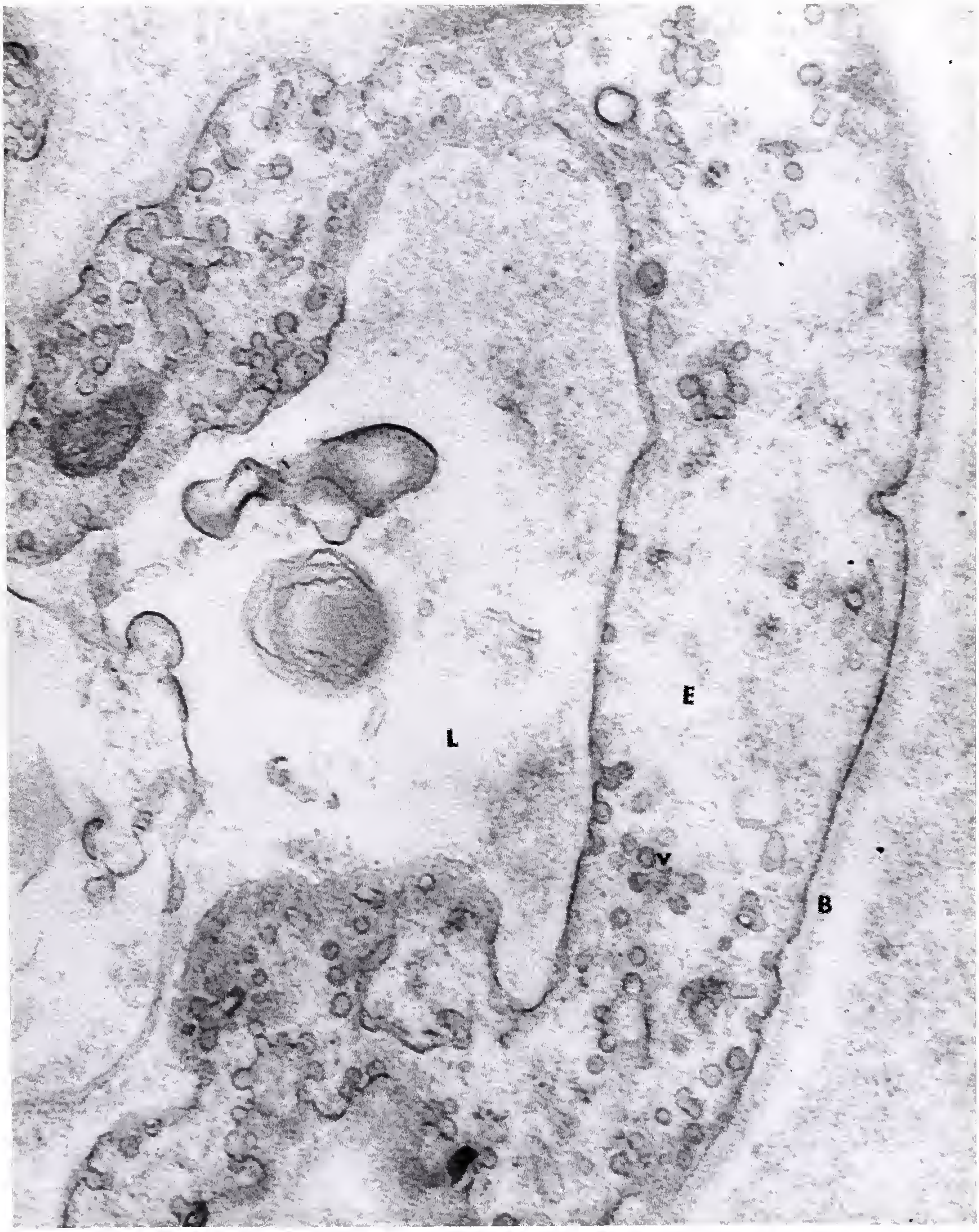


Fig. 12. Control Preparation Electron micrograph of part of a capillary wall taken from tissue incubated in medium without substrate. Numerous vesicles (v) are present in the cytoplasm of the endothelial cell (E). B marks the basement membrane and L the vessel lumen.

x44, 000


E
L
V
B

Electron micrographs of blood vessels in heart and lung incubated in complete ATP medium are shown in figs. 13-15. The final product of the enzymatic reaction was clearly localized only to the pinocytic vesicles of the endothelial cells. In some instances the vesicles were filled with reaction product, and in other cases the reaction product was deposited on or adjacent to the inner surfaces of the vesicle membrane. Activity was not seen free in the cytoplasm of the endothelial cells nor on the plasma membranes (both luminal and extravascular) when they were present as linear structures. However, when the vesicles were continuous with the plasma membranes these sites showed enzymatic activity (figs. 14, 15). No reaction product was seen in other organelles of the endothelial cells, at the sites of intercellular junctions, or in the basement membrane. In the lung, reaction product was also seen in vesicles of the alveolar lining cells (fig. 15) and reaction product was regularly seen bordering the surfaces of red blood cells (fig. 15).

Blood Vessels Lined by Continuous Endothelium with a Continuous Glial Investment

Blood vessels of the retina were studied as examples of type II vessels. ATPase activity was present in the small blood vessels of the retina as demonstrated by light microscopy (figs. 16, 17) but the resolution was inadequate for determining which structures of the wall

Fig. 13. ATP Reaction Electron micrograph of part of capillary wall of myocardium incubated with ATP. The dense reaction product is confined entirely to small localized sites in the cytoplasm of the endothelial cell (E). Part of a muscle cell is seen at the right (M).

x39, 000

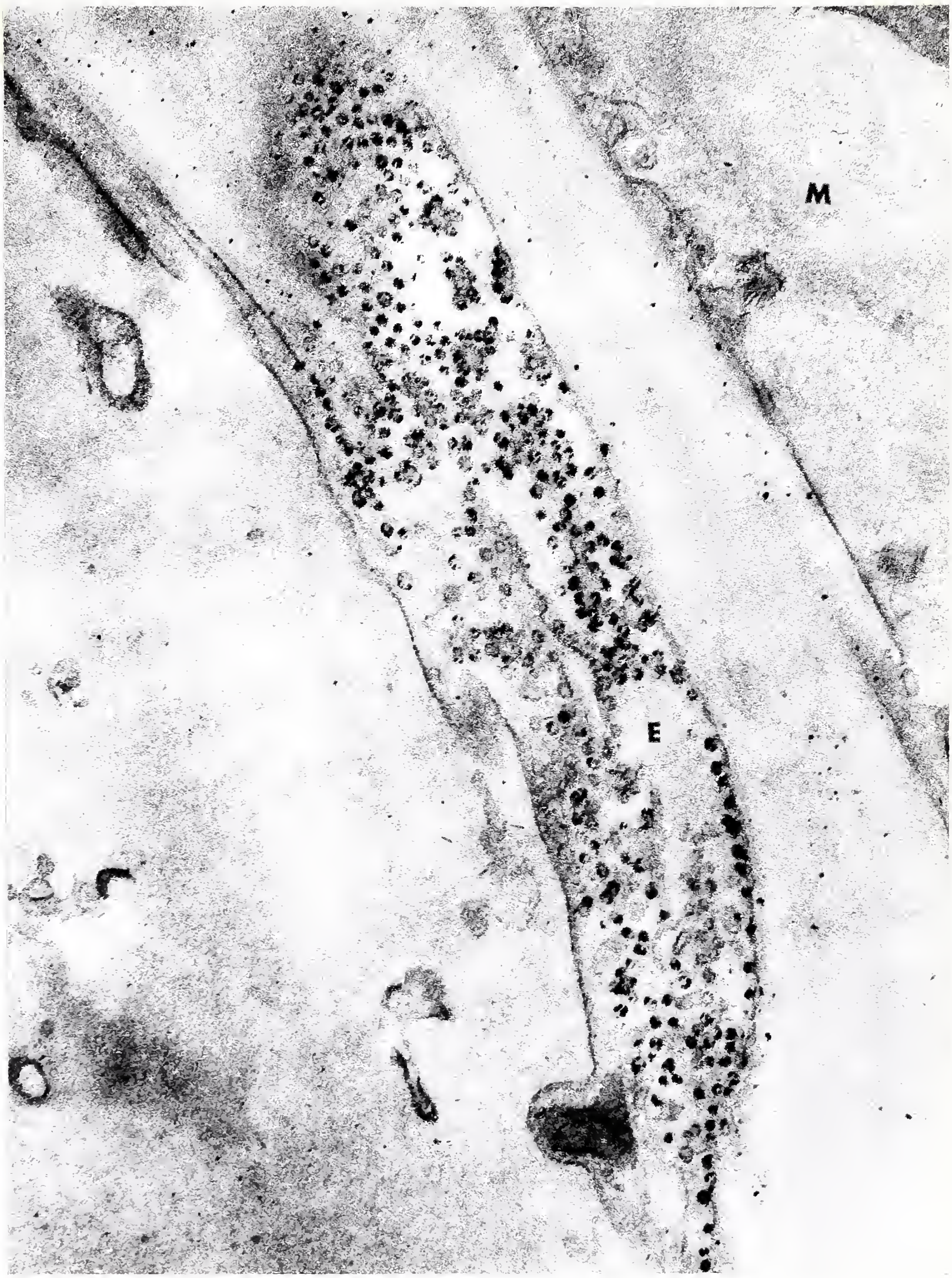

M
E

Fig. 14. ATP Reaction Part of the endothelial cell seen in fig. 13. Numerous vesicles (v) with reaction product are open to the extravascular side of the endothelial cell. No reaction product is seen on the plasma membranes where vesicular indentations do not occur. The reaction product appears to be on the inner surface of the membrane and inside the vesicles. No reaction product is seen in the basement membrane (B).

x83, 000

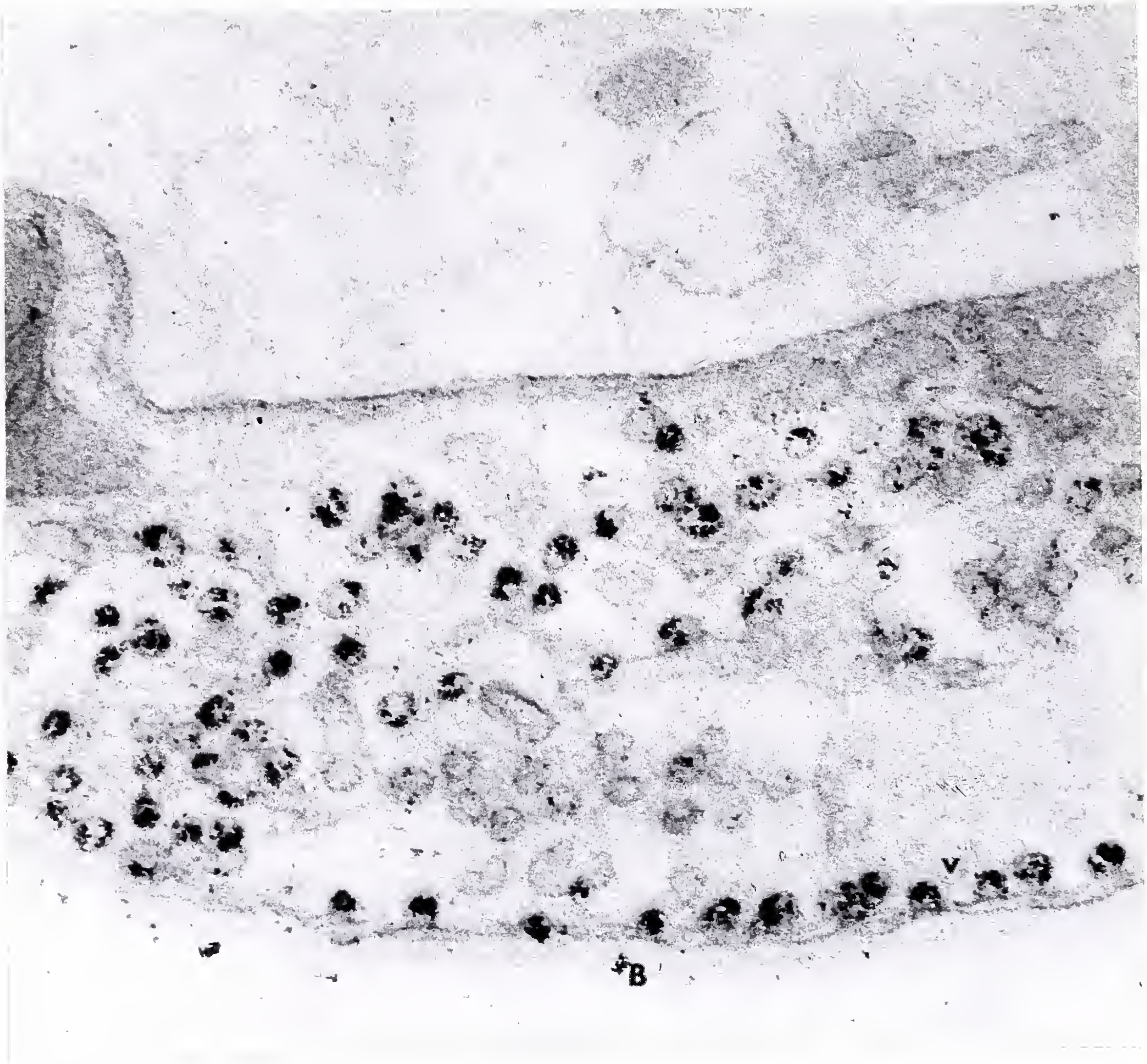
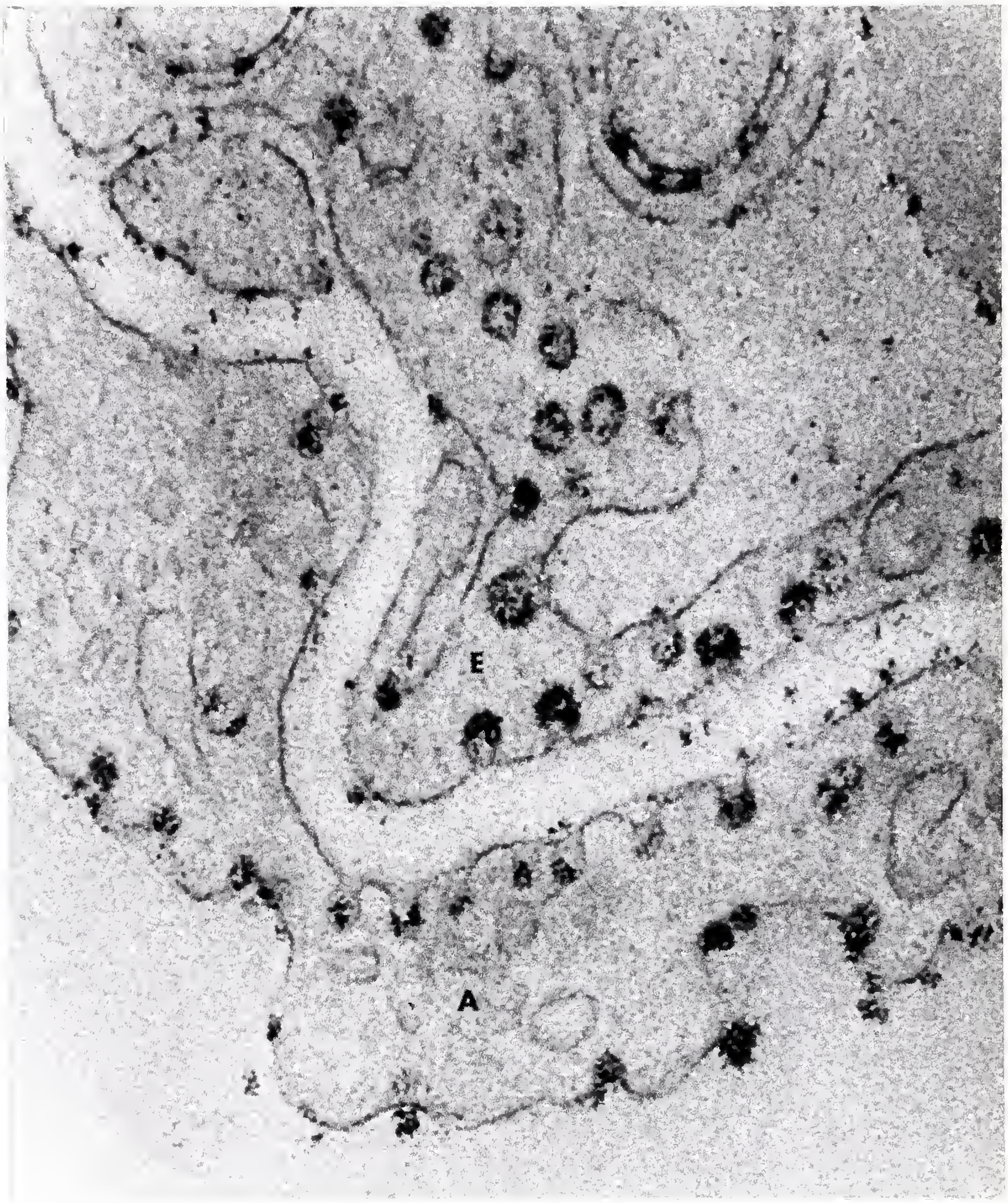

V
B

Fig. 15. ATP Reaction Part of a capillary wall (E) and alveolar lining cell (A) of a lung incubated with ATP. Reaction product is confined to the pinocytic vesicles of the endothelial cell and is also seen in vesicles of the alveolar lining cell.

x140,000

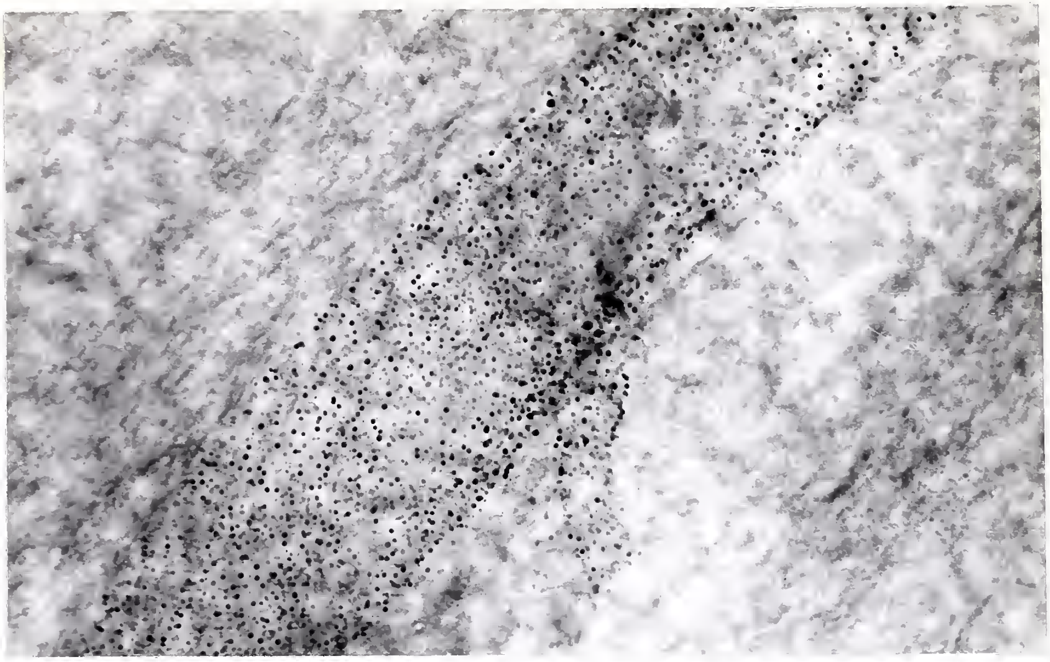

E
A

contain the enzymatic activity. Control studies included incubation of retina in medium containing lead alone and in medium with BG substituted for ATP. No evidence of enzymatic activity or non-specific lead adsorption was present in the control preparations.

Fig. 18 is an electron micrograph of a blood vessel in retina incubated with BG. The structure of this vessel is typical of the vessels in the retina (and central nervous system) as described by other workers employing conventional electron microscopic techniques. The vessel wall is lined by a continuous layer of endothelium and basement membrane. Outside the basement membrane numerous glial cell processes are joined together to form a dense cellular network. The extracellular space is limited to the small (100-250Å) gaps between the cell membranes of the glial cells.

Electron micrographs of retina incubated in ATP medium are shown in figs. 19-22. The most striking finding was the presence of reaction product on the surface membranes of the glial cells. Reaction product in the form of a thin, dense precipitate filled the interspaces between the apposed glial cell membranes. Reaction product was also seen on the glial membranes which surround the basement membranes of the blood vessels figs. 19, 20, 21. In some cases reaction product was deposited in the basement membrane region of the blood vessels (figs. 20-22). It is not clear whether this represents

Fig. 16 and Fig. 17. ATP Reaction Light photomicrographs of flat preparations of retina incubated in standard ATP medium. Reaction product is localized to the walls of the small blood vessels.



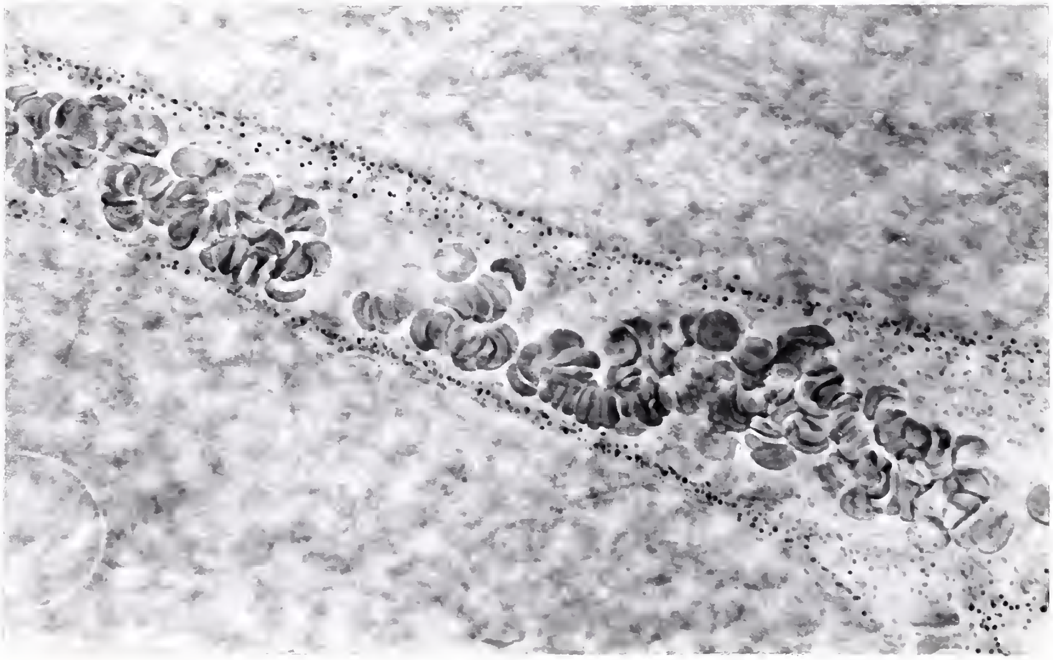


Fig. 18. Control Preparations. Electron micrograph of retina incubated with beta glycerophosphate and buffered at pH 7.1. Under these conditions the small blood vessels and glial cell membranes are unreactive. E marks the endothelium, G some of the surrounding glial cells.

x25,000

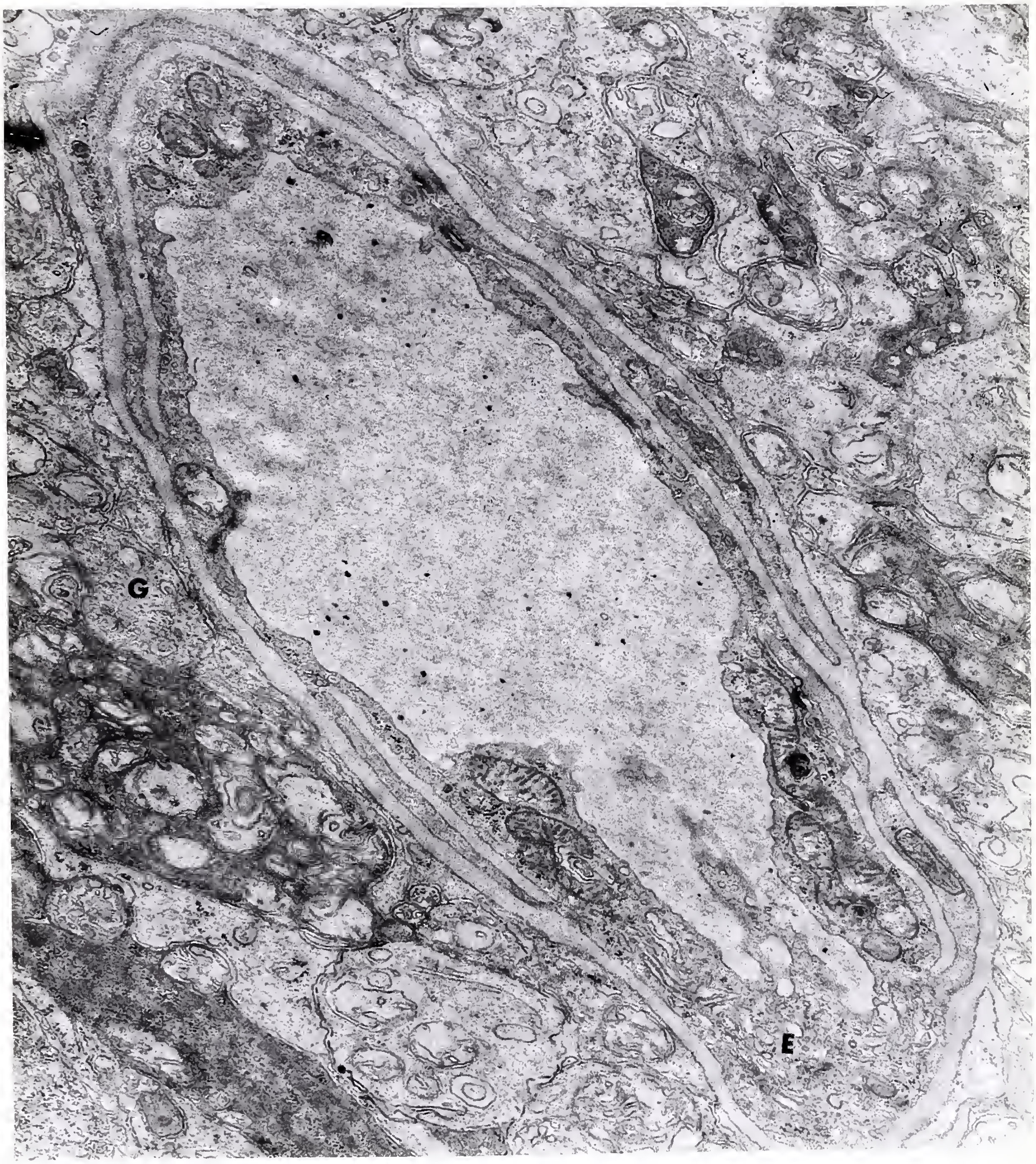

G
E

Fig. 19. ATP Reaction. Electron micrographs of retina incubated in standard ATP medium Dense reaction product lines the membranes of the glial cells (G). No reaction product is seen within the endothelial cells (E) of a small capillary. L marks the capillary lumen.

X16, 000

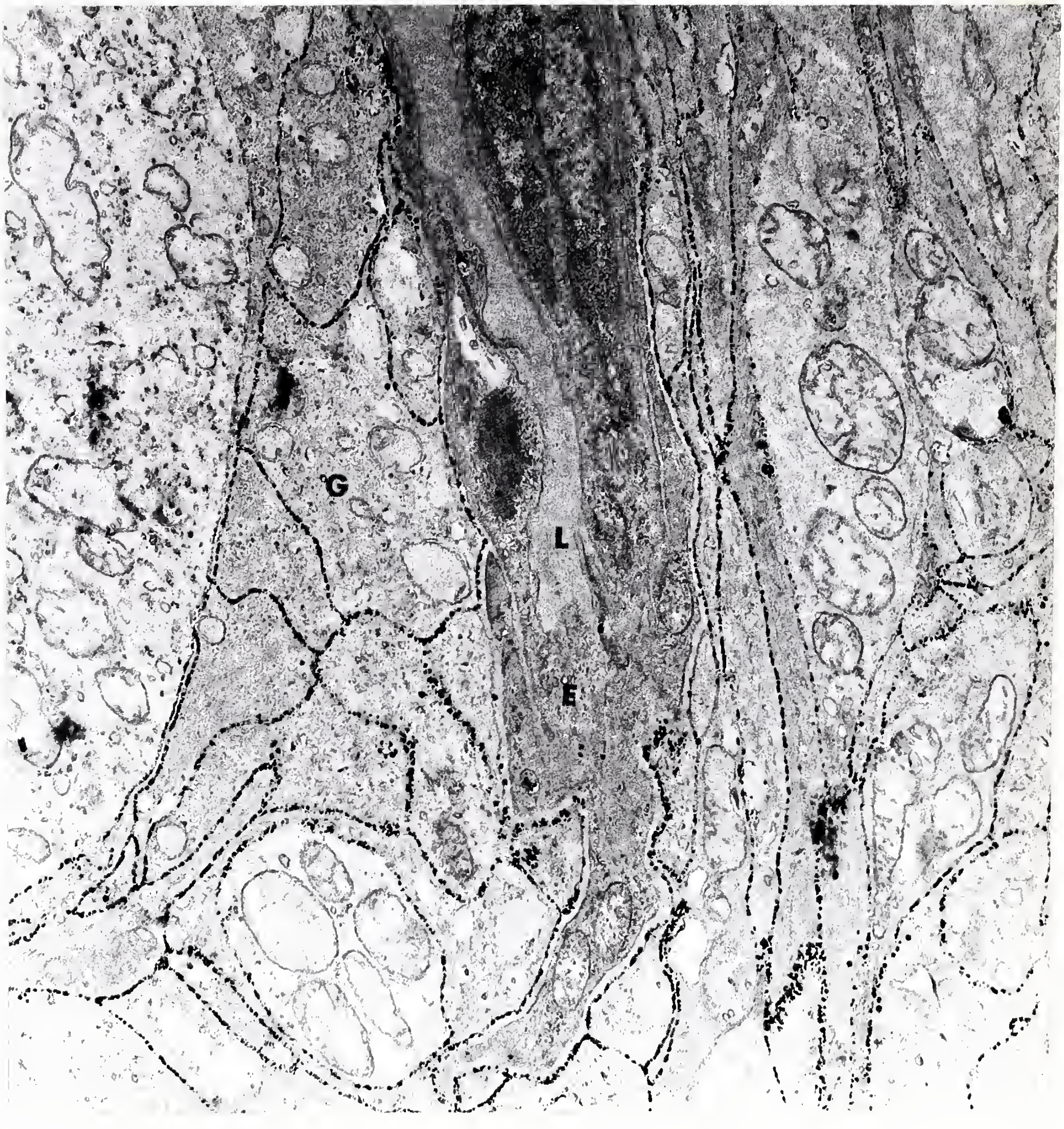

G
L
E

Fig. 20. ATP Reaction. Part of retina from same preparation shown in fig. 19. Reaction product is localized to the space immediately surrounding the endothelial cells (E). Part of this is seen at higher magnification in fig.21. L marks the vessel lumen.

x32, 000

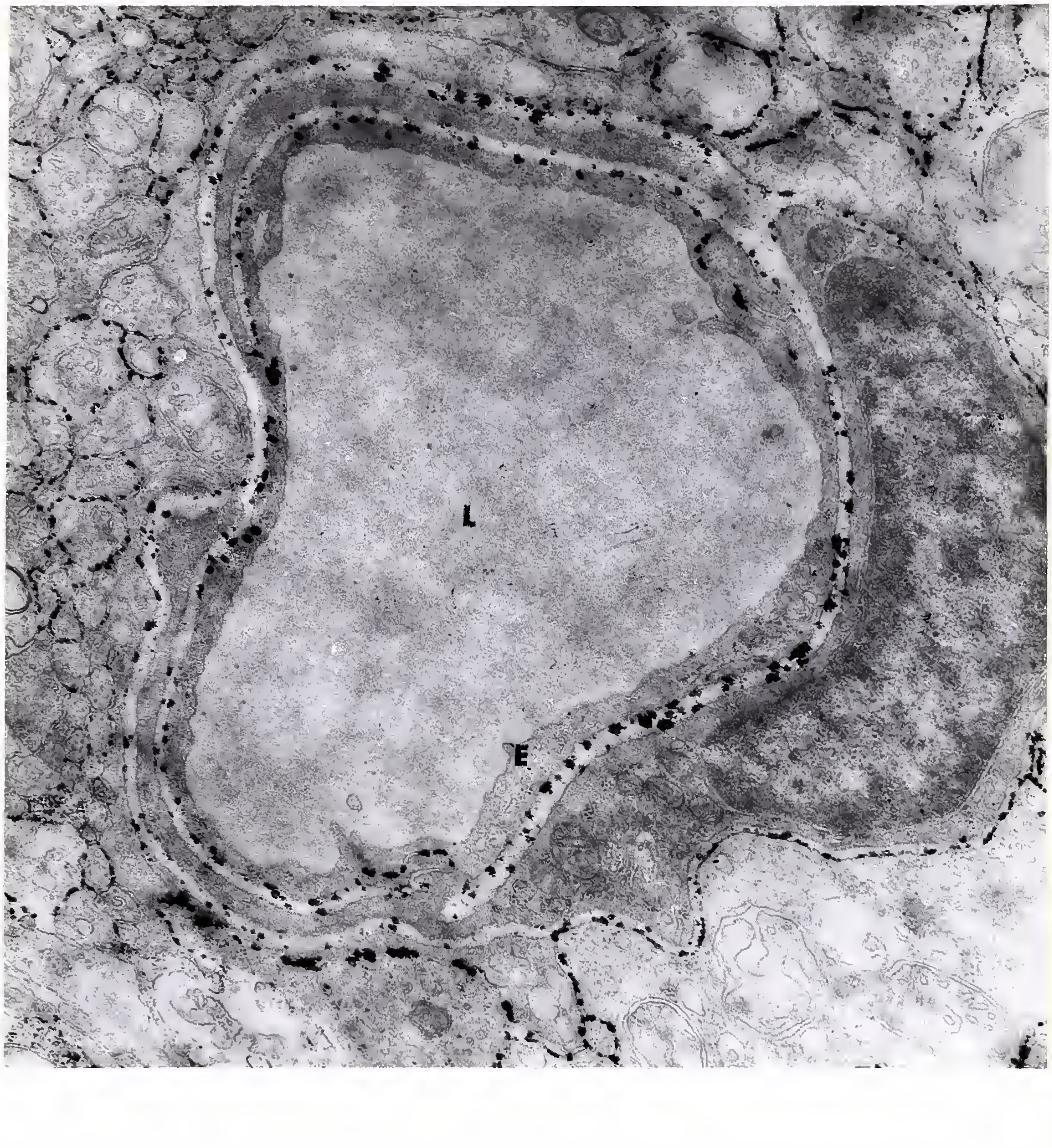

L
E

Fig. 21 ATP Reaction Part of fig. 20. Reaction product in the basement membrane "space" appears to be localized to the outer membranes of the endothelial cell (E) and the membranes of the adjacent glial cells (G). No reaction product is seen within the endothelial cells nor on the membranes of the luminal surface.

x44, 000

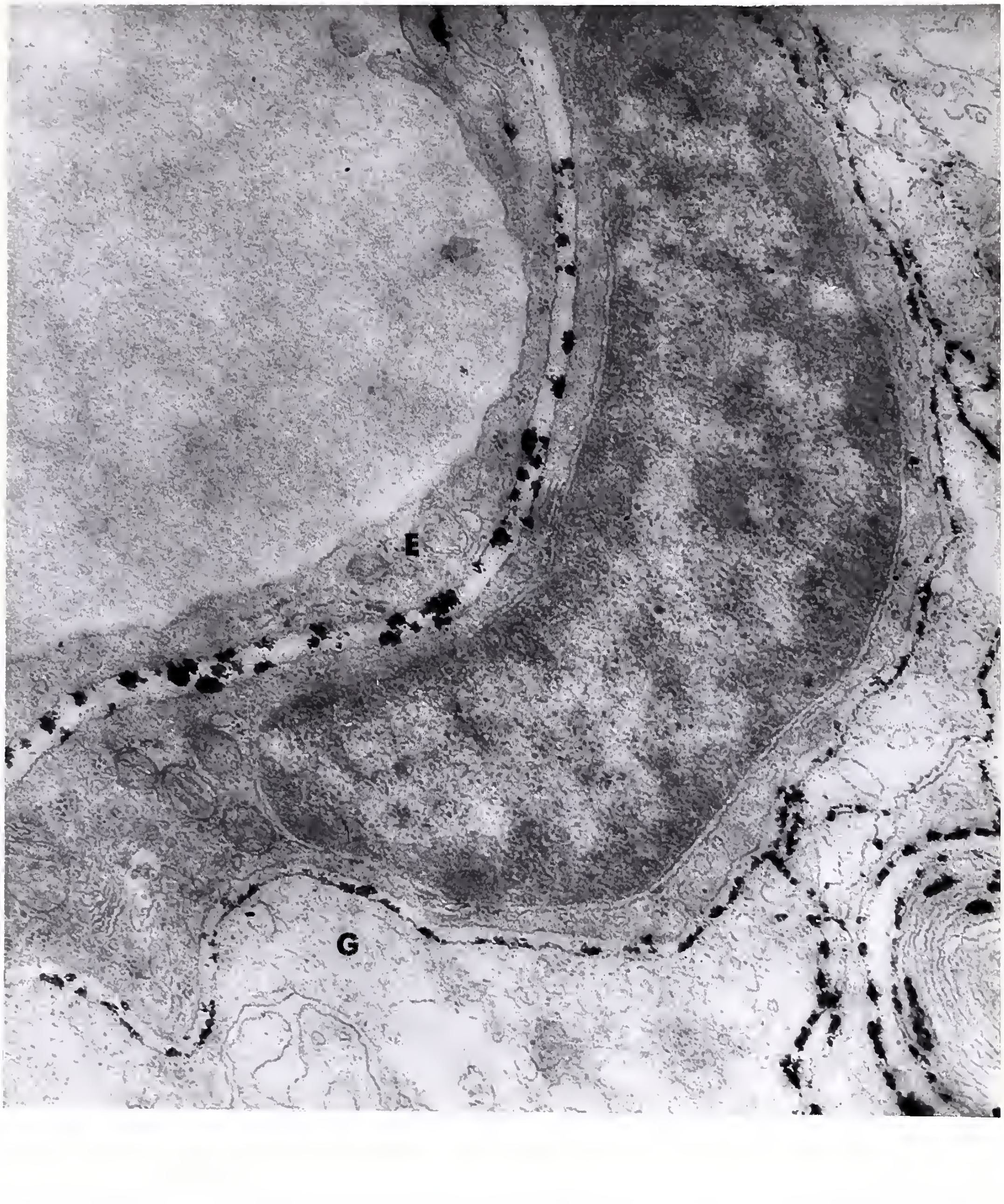
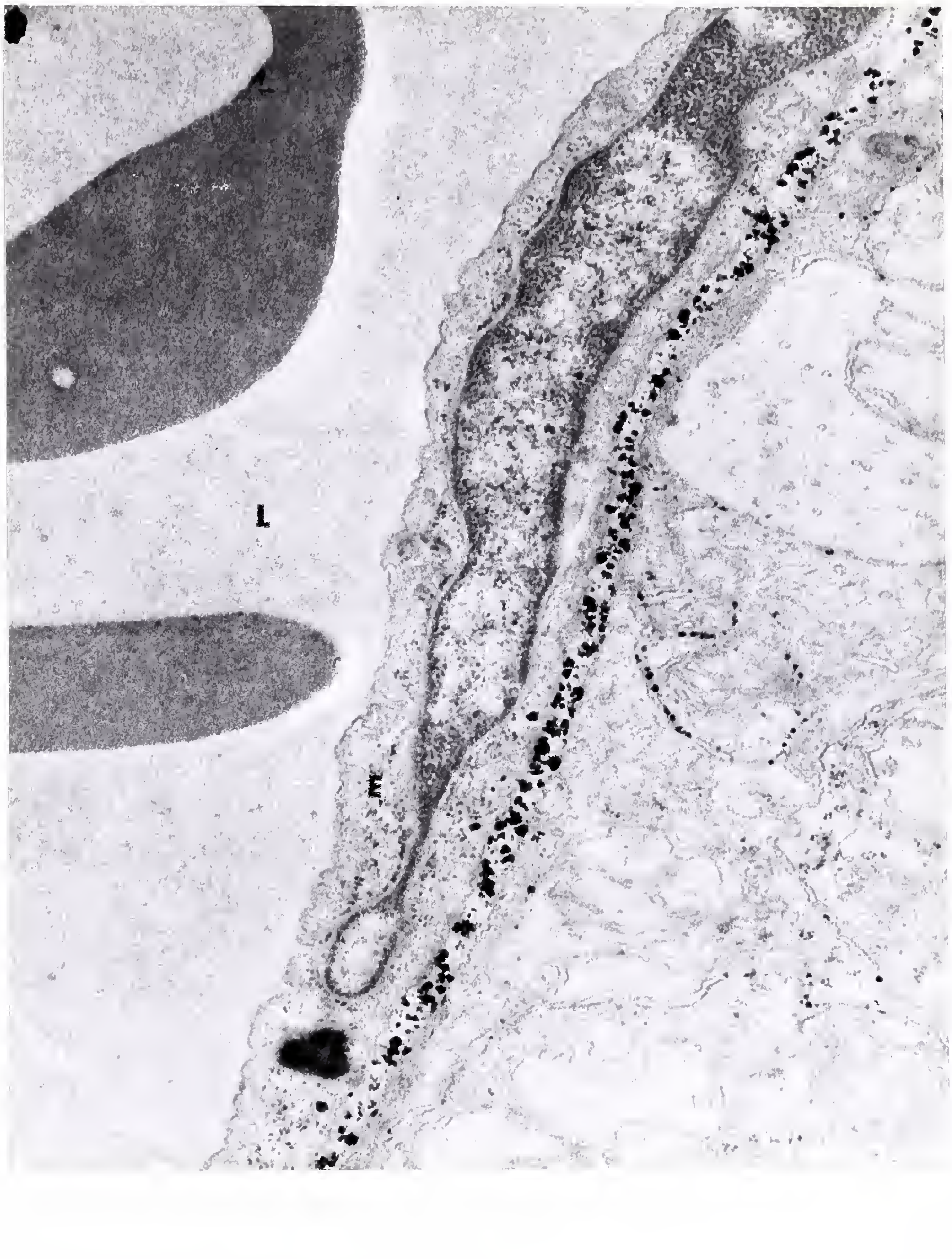

E
G

Fig. 22 ATP Reaction Electron micrograph of retina incubated in standard ATP medium. Part of a venule is seen with dense reaction product confined to the basement membrane region. E marks the endothelium and L the vessel lumen.

x 32,000


L
E

accumulation of reaction product which originated from enzymatic activity on the membranes of the glial cells (or possibly the extravascular membranes of the endothelial cells). It is also conceivable that this deposition indicates that the enzymatic activity is present in the basement membrane itself.

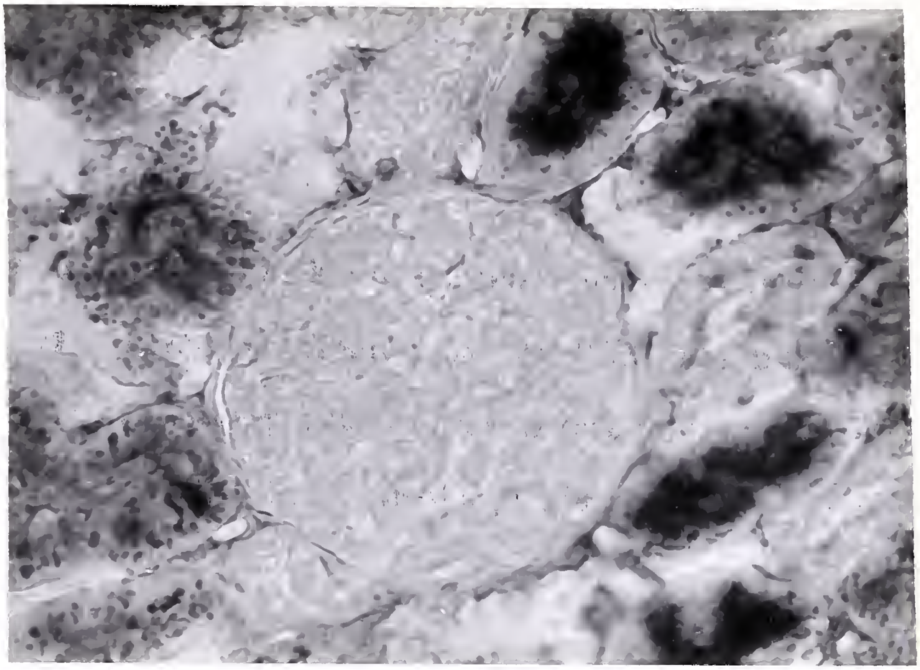
While the basement membrane localization of reaction product might reflect the presence of enzymatic activity on the extravascular membranes of the endothelial cells, there is no indication that any activity is present either within the endothelial cells or on the cell membranes of the luminal surface. No reaction product was seen within the few pinocytic vesicles found in these endothelial cells.

Blood Vessels Lined by Endothelium with Intracellular Fenestrations

Glomerular and peritubular capillaries of the kidney and blood vessels in small intestine were studied as examples of type III vessels. The presence of ATPase activity in the glomerulus as viewed with the light microscope is shown in fig. 24. Control studies as described for the other vessels were also carried out on these vessels with the same results; ATP was hydrolysed by the glomerular capillaries, BG was inactive, and lead did not adsorb non-specifically.

Fig. 23 Control Preparation Light photomicrograph of a frozen section of kidney incubated with beta glycerophosphate and buffered at pH 7.1. No reaction product is visible in the glomerulus. Reaction product is present in the surrounding tubules.

Fig. 24. ATP Reaction Light photomicrograph of a frozen section of kidney incubated with ATP and buffered at pH 7.1. Dense reaction product is seen within the glomerulus, and reaction product is also present bodering the tubules.



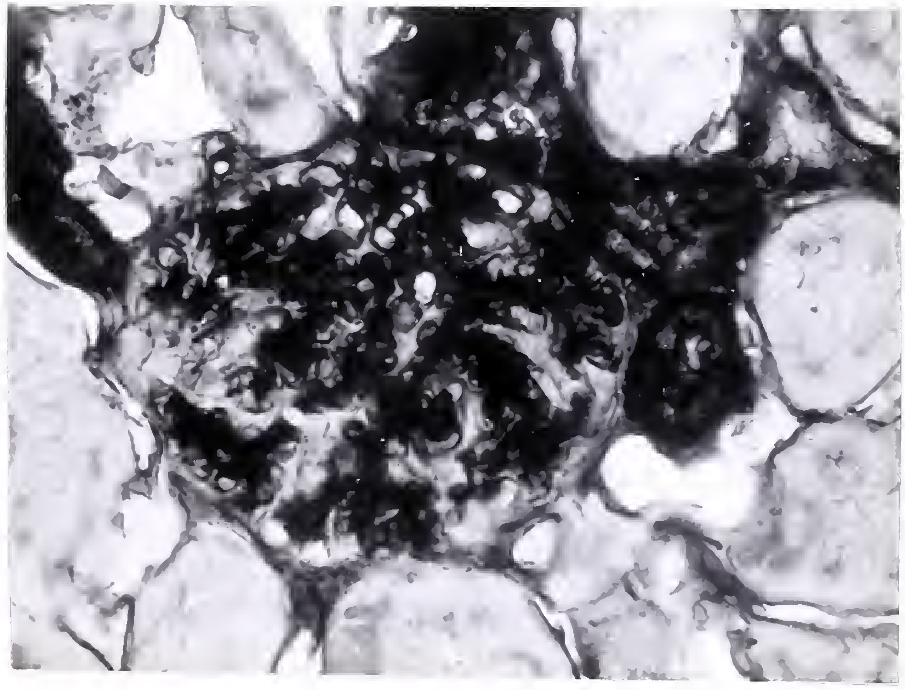


Fig. 29 is an electron micrograph of a capillary in small intestine incubated in ATP medium. The fenestrated endothelium of this vessel is also unreactive, and no activity was seen on the basement membrane or the surrounding periendothelial cells.

Electron micrographs of kidney incubated with ATP are shown in figs. 25-28. In the glomerulus, reaction product was confined to the epithelial cells (figs. 25-27). No reaction product was seen on the fenestrated endothelium or its basement membrane. At higher magnification (fig 27) the reaction product on the epithelial cells was localized to the outer membranes of the cells and their foot processes. No reaction product was seen within vesicles of the epithelial cells or other cytoplasmic structures.

A study of peritubular capillaries of the kidney showed that the fenestrated endothelium of these vessels was also unreactive (fig. 28). The basal infoldings of the tubule cells were reactive as were the membranes of the red blood cells.

Discussion

Differences have been found in the localization of ATPase activity in small blood vessels of normal rats. Blood vessels lined by continuous endothelium with pinocytic vesicles (type I) showed activity only in the pinocytic vesicles and on the cell membranes which form the vesicles. In vessels lined by continuous endothelum with a continuous glial investment (type II) activity was present in the basement membrane region of the blood vessels and on the outer membranes of the glial cells and their processes. No activity was present either within the endothelial cells or on the cell membranes bordering the luminal

Fig. 25. ATP Reaction Electron micrograph of part of a glomerular tuft from kidney incubated in ATP medium. Dense reaction product is localized to the foot processes and membranes of the epithelial cells, but no activity is seen in the capillary endothelium (E). Part of this area is seen at higher magnification in fig. 26.

X16, 000

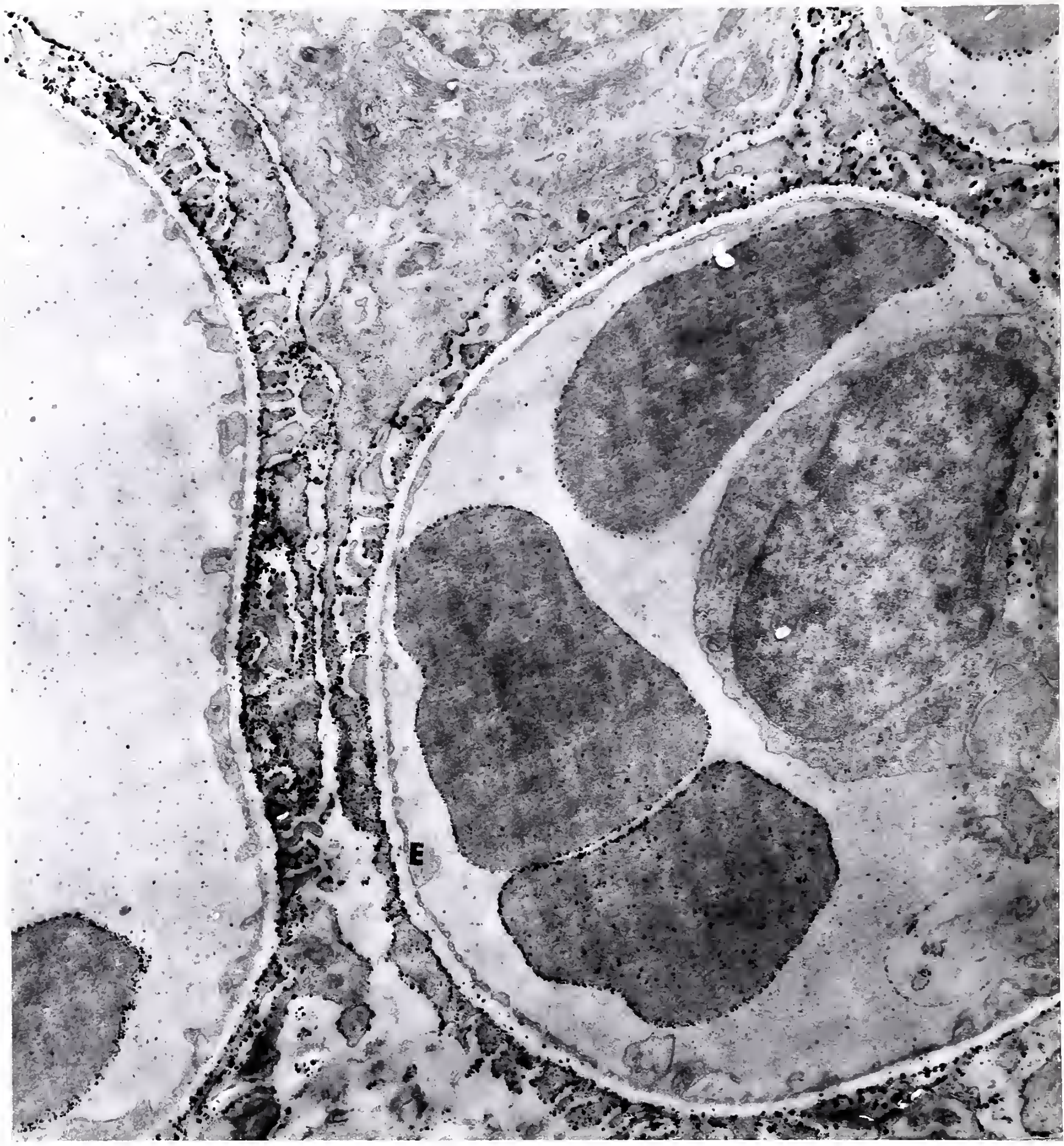

E

Fig. 26. Part of the glomerulus seen in fig. 25. The fenestrated endothelium (E) and basement membrane (B) of the glomerular capillary is unreactive. Dense reaction product lines both the red blood cells (R) and the epithelial foot processes (P).

X36, 000

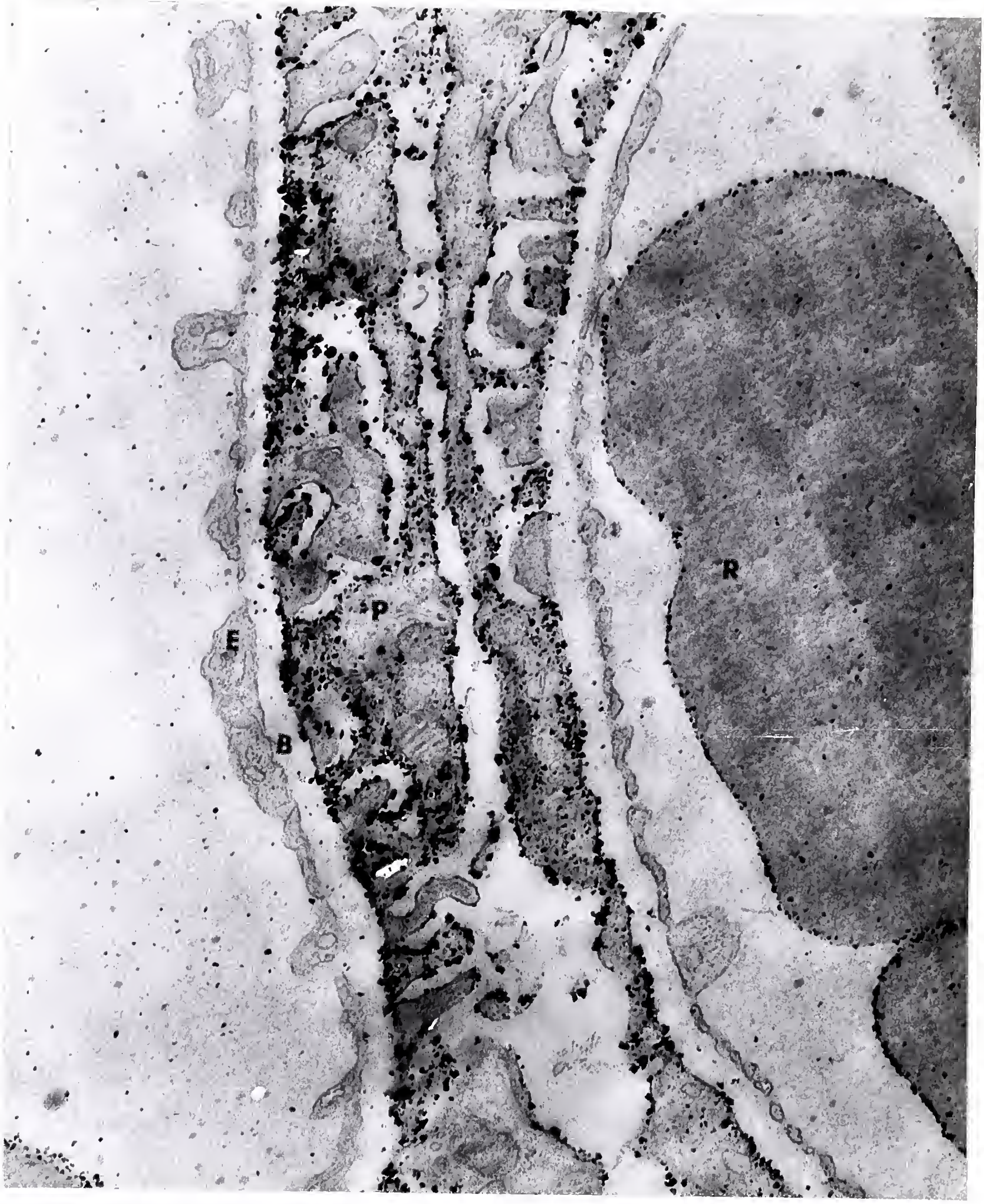

R
P
E
B

Fig 27. ATP Reaction Part of a glomerulus from the same preparation shown in fig . 25. The reaction product is localized to the outer membranes of the epithelial cell foot processes.

X55, 000

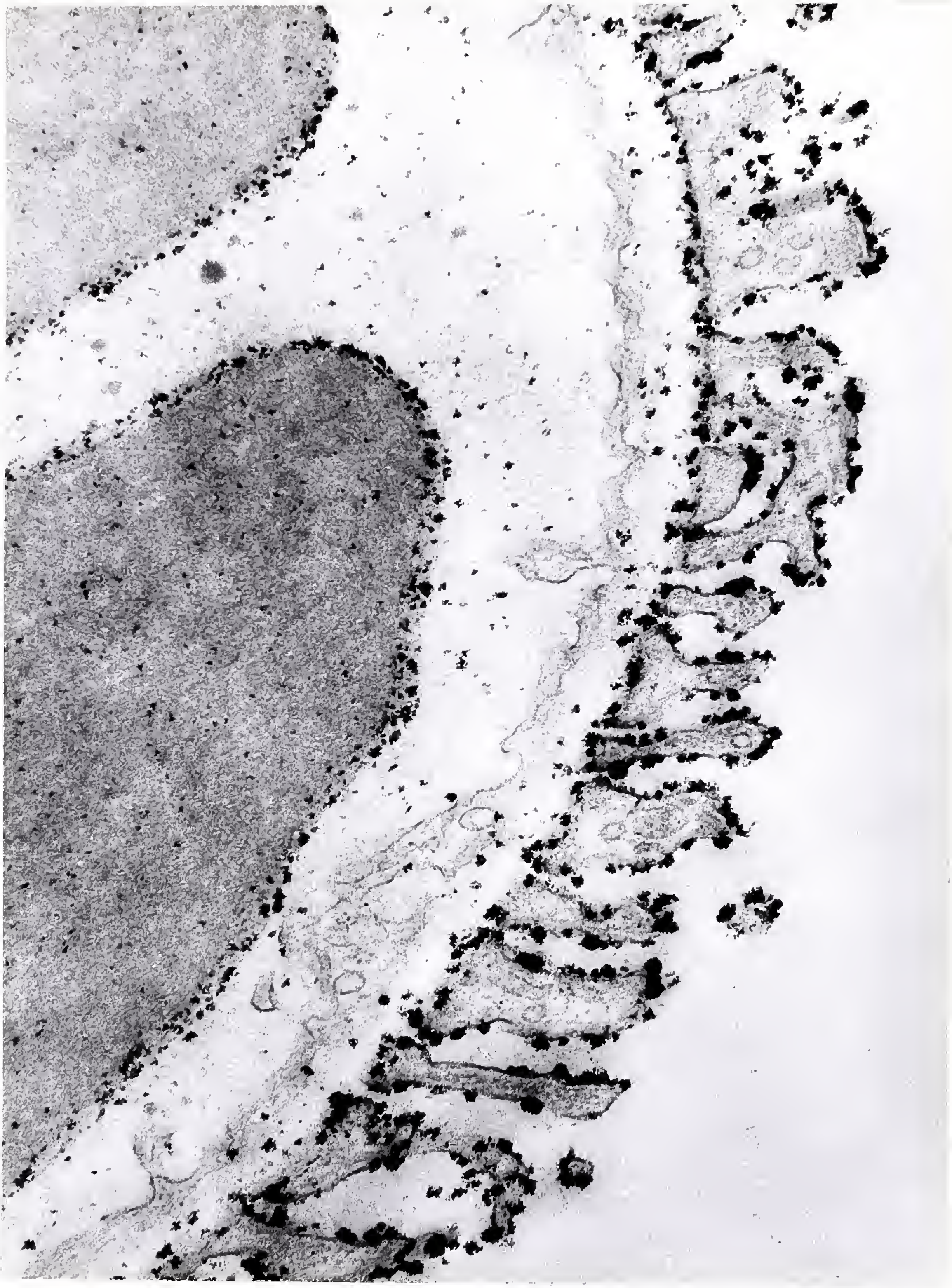


Fig. 28. ATP Reaction Electron micrograph of kidney incubated in standard ATP medium. Part of a peritubular capillar is seen with a red blood cell (R) in the lumen. Reaction product lines the border of the red cell while the fenestrated endothelium (E) lining the capillary is unreactive. The extensive infoldings of the basal borders of the tubules are also reactive.

x44, 000

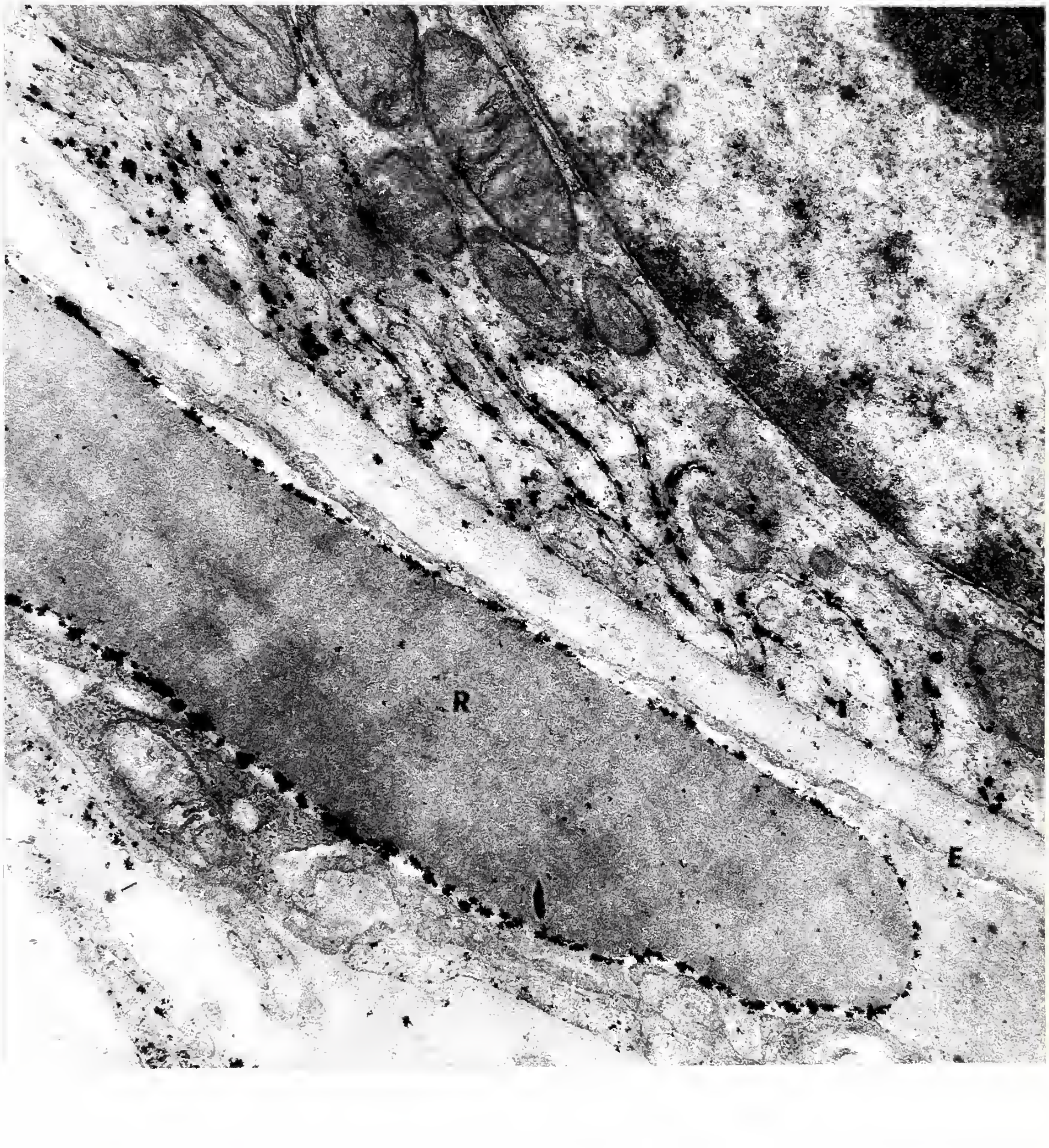
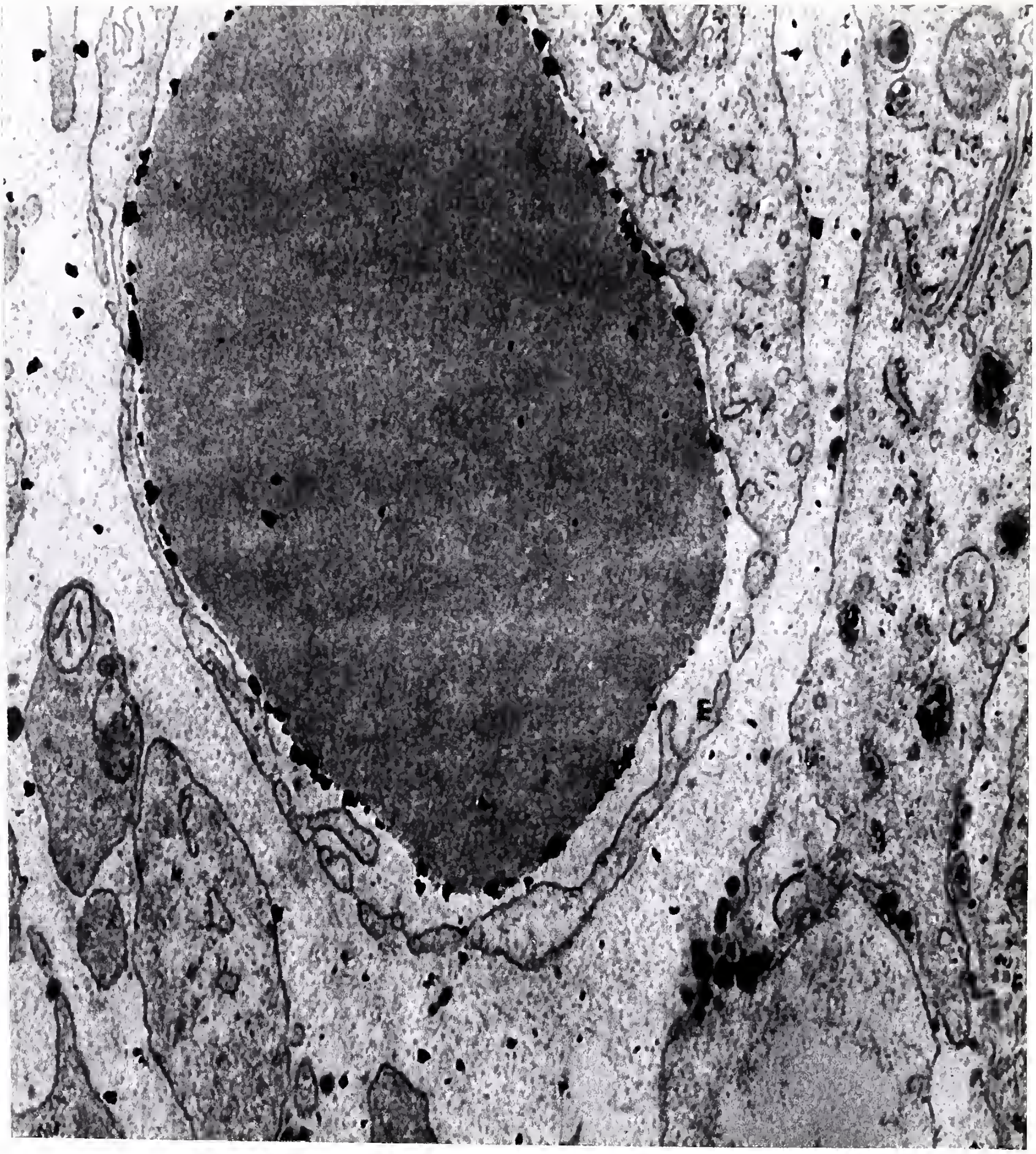

R
E

Fig. 29. ATP Reaction Electron micrograph of small intestine lamina propia incubated in standard ATP medium. Part of a capillary is seen with a red blood cell in the lumen. Reaction product is present on the membrane of the red blood cell but the fenestrated endothelium (E) of the capillary wall is unreactive.

X44,000


E

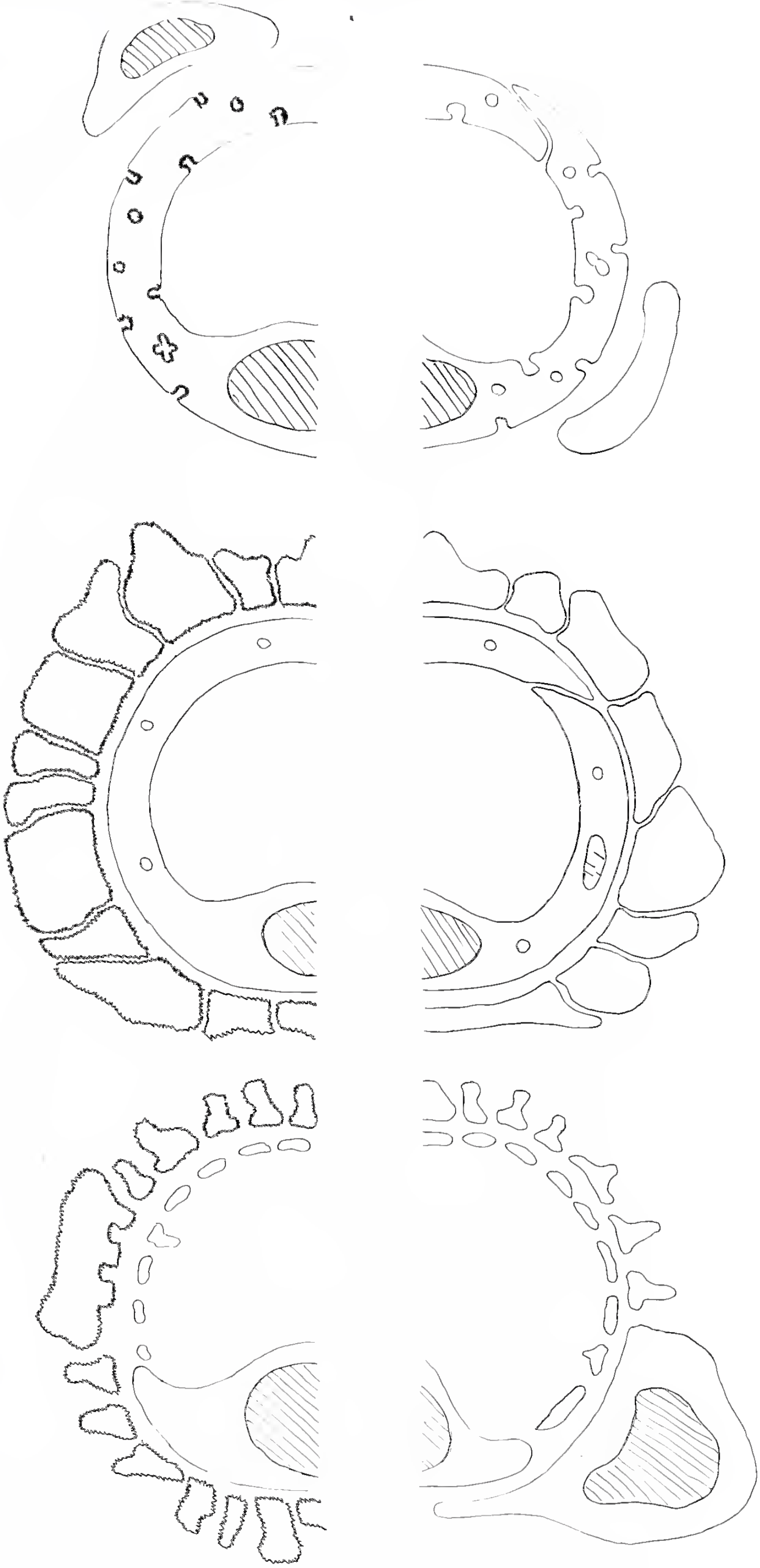
surface. In vessels lined by endothelium with intracellular fenestrations (type III) activity was not found in the endothelial cells or the basement membranes. In the glomerulus activity was present on the outer membranes of the epithelial cells and their foot processes. These findings are summarized in diagramatic form in fig. 30.

In considering the possible significance of these findings three general problems must be evaluated:

1. technical limitations of the methods
2. biochemical specificity of the enzyme (s) studied
3. possible physiological function

Technical Limitations of the Methods

With the techniques of conventional histochemistry at the level of the light microscope, enzymatic activity could be localized to specific tissues and cell types with reasonable accuracy. The localization of activity within cells or to specific subcellular sites has been less precise, and when it was possible this was confined to large organellels in certain cell types (eg. mitochondria in heart muscle). The application of histochemistry to electron microscopy offered the possibility of localizing enzymatic activity to specific subcellular sites, provided the problems of fixation and reaction product recognition could be solved (56).



30

The problem of fixation involved finding substances which would provide adequate preservation of fine structure for electron microscopy while still retaining the enzymatic activity of the tissue. Osmium tetroxide, a most suitable fixative for electron microscopy, was not suitable for electron histochemistry since adequate fixation resulted in complete loss of enzymatic activity (56). This property has been used to inactivate tissue before incubation and served as a control to rule out nonspecific chemical interactions of substrate and capture reagents with the tissue.

Fixatives conventionally employed for light microscopy have been modified for use in electron microscopy with some success (57, 58). Recently, new aldehyde fixatives have been developed which provided good structural preservation of the tissue while still retaining enzymatic activity (59). One of these new fixatives, glutaraldehyde, has been used extensively in this study. It was found, however, that not all enzymes remained active following the use of this fixative (59) and therefore the absence of activity at some sites in tissues might be due to the selective inhibition of the enzymes by the fixative. In this regard the localization of ATPase in the pinocytic vesicles of heart capillaries was also studied in tissues fixed in hydroxyadipaldehyde (59) and formalin (58), and the localization of the activity was the same in each case. These additional fixation controls have

not been carried out on the other tissues reported in this study.

The problem of reaction product recognition was solved in this study by the use of the Wachstein-Meisel method (60) for the demonstration of adenosinetriphosphatase (ATPase) activity. In this method a lead salt was the capture reagent and the reaction product was an insoluble lead precipitate. This precipitate was electron dense and readily visible in the electron microscope.

However there have been some recent objections to the use of lead salt methods on small tissue blocks for electron microscopy (61, 62). Novikoff et al (61) and Holt and Hicks (62) claimed that heavy metal reagents and smaller molecule substrates diffused into tissue blocks at different rates, and this, they suggested, might have resulted in false localization of reaction product. To eliminate this possibility they suggested that 50u frozen sections of tissue were more suitable for incubation (tissue destruction from freezing and thawing notwithstanding) than small blocks of tissue.

To rule out the possibility of false localization in the present study both frozen sections and small blocks from a numer of tissues were incubated in parallel; the localization of reaction product was the same in both preparations, but the structure of the tissue incubated as small blocks was far better than in the frozen sections.

An additional problem related to the use of lead salt methods for enzyme localization was the possibility of developing too extensive a precipitate which could obscure the site of origin of the reaction. In the case of the retinal vessels dense reaction product filled the basement membrane space in some of the micrographs, and it was not clear whether the enzymatic activity was on the membranes of the endothelial cell, the glial cells, the basement membrane, or all three. Shorter incubation times with lower substrate concentrations might clarify this point.

Biochemical Specificity

The determination of the biochemical specificity of enzymes by histochemical techniques is a formidable problem. Since the aim of the histochemical approach is to analyse the chemical activity of cells and subcellular sites in situ, the enzymes under study are not (and usually cannot be) assayed in the isolated state.* This eliminates the possibility of characterizing the enzymes in terms of their kinetics and quantitative reactions to activators and inhibitors. Instead enzymes must be studied in terms of their activity toward

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* Parallel histochemical and biochemical studies have been carried out on some tissues (58). With tissues such as small blood vessels which are not isolated easily by conventional biochemical techniques, histochemical information is the only data available.

different substrates and their qualitative response to activators and inhibitors.

In the present study the sites in blood vessels which were reactive when incubated with ATP were not reactive when BG was substituted for ATP. Other nucleoside phosphates have also been found to be hydrolysed by enzymes in blood vessels and other nonnucleoside phosphates have been negative at the light microscope level. (63) This indicates that the enzymatic activity in the blood vessels is probably a nucleoside phosphatase rather than a single ATPase or a non-specific alkaline phosphatase.

An attempt has also been made to characterize the activity of the ATPase in blood vessels in terms of its activation by sodium (Na), potassium (K), and magnesium (Mg), and inhibition by Ouabain, p-chloromercuribenzoic acid, and n-ethyl maleimide (63). These studies were stimulated by many recent biochemical studies of ATPase activity in other systems in which the ATPase was activated by Na, K, and Mg and specifically inhibited by Ouabain (referred to in introduction).

The results of these studies have been negative or at best non-contributory. No significant activation or inhibition of the enzymatic activity in blood vessels by these agents was detected. Other workers have made similar attempts along these lines with the same negative results (51).

It should be stressed that these negative findings only indicate that the enzymatic activity in the blood vessels could not be totally inhibited by these agents; partial inhibition of the activity (which might be expected if the inhibition is competitive) would not be detected by the techniques used.

It has also not been determined whether the enzymatic activity found at different sites in different blood vessels is due to the same or different enzymes.

In summary the rather meager information on the chemical specificity of the ATPase demonstrated in blood vessels indicates that the activity is probably due to one or several nucleoside phosphatases.

Physiological Significance

Although the chemical specificity of the enzyme (s) demonstrated in this study has not been clearly determined, the localization of reaction product in the different blood vessels remains a demonstrated fact, and the evidence indicates that the activity marks the sites of utilization or hydrolysis of ATP. In an attempt to speculate on the significance of these findings some of the known pathways of ATP utilization should be considered.

1. ATP is involved in a multienzyme system and serves to provide high energy phosphate bonds for some part of the reaction
2. ATP is used in the formation of phosphatidic acid (64, 65) which might function in the transport or sodium across cell membranes or play a part in membrane synthesis.
3. ATP is used in the formation of phosphoproteins (66) which may serve as ionic carriers across cell membranes.
4. ATP is utilized by the Na-K-Mg activated ATPase system which is involved in the transport of Na and K across cell membranes (67)
5. ATP is a source of ADP which serves to regulate cell metabolism (68).

Activity in Pinocytic Vesicles

Since the pinocytic vesicles of type I blood vessels are sites of special membrane activity it is possible that the ATPase activity in them is involved in the formation or activation of cell membrane, perhaps through a phosphatidic acid mechanism. Also if one considers that the sequestered material within the vesicles includes salts and water having the composition of extracellular fluid it is also possible that the ATPase on the vesicle membrane serves to maintain the electrolyte differences between the vesicle and cell cytoplasm. In this case the enzyme might function as a cation pump similar to the Na-K-Mg ATPase demonstrated biochemically in other tissues.

It has been suggested that sites on the cell membrane which form vesicles might bind certain substances. This would result in a high concentration of these substances in the vesicles and this might provide a more efficient and perhaps selective transport mechanism (24). In this hypothesis the ATPase might be involved in an activating reaction.

The absence of enzymatic activity on those portions of the endothelial cell membranes which do not form vesicles is difficult to interpret, since it might be argued that the enzymes there are inhibited by the fixative. But since the same localization of activity was obtained with three different fixatives, it is also possible that the vesicles represent a special kind of cell membrane.

Activity on Glial Cell Membranes

Some investigators consider that the minute (100-250Å) gaps between apposed glial membranes constitute the extracellular space of the retina and central nervous system (69). If this is so, and some recent experimental evidence supports this view (70), then the glial membranes would require a pumping mechanism to maintain the electrolyte differences between the cells and the extracellular space. Furthermore it has been suggested that the differences in the calculated volumes of extracellular space in these tissues arrived at by physiological and morphological data (70) could

be explained if the extracellular fluid in the brain and retina had a higher concentration of electrolytes than the extracellular fluid in other tissues (71). Carrying this hypothesis further, the ATPase activity lining the membranes of the glial cells might function as the pump mechanism to maintain this higher gradient. Similar enzymatic activity has also been demonstrated on the glial membrane in the central nervous system (77).

On the other hand, since glial tissue forms a solid investment around blood vessels and is therefore interposed between the blood stream and the neurones it is also conceivable that the enzymatic activity on the glial membranes is concerned with the facilitation of the transport of substances across the glial cells to the neurones.

Activity on Epithelial Cell Membranes

Recent studies (37, 38) have indicated that the foot processes of the epithelial cells which line the basement membranes of the glomerular capillaries may function to monitor the passage of materials across glomerular capillaries. It has been shown that tracer particles, injected into the blood stream of animals, passed across the fenestrated endothelium readily but were held up by the basement membrane. Particles which eventually traversed the basement

membrane were taken up into the epithelial cells by membrane vesiculation. In the light of these findings it is possible that the ATPase activity demonstrated on the epithelial cell membranes may be involved in this process. These cells are also thought to be involved in the selective uptake of large molecules and the maintenance of electrolyte gradients, and in both instances known pathways of ATPase reactions could be functional.

The Absence of Enzymatic Activity

Discounting a possible technical explanation for the absence of enzymatic activity at any particular site, the distribution of activity in the different blood vessels bears some relationship to the type of endothelial cell lining. Where the lining is not a continuous morphological barrier no enzymatic activity is evident, although some would dispute the contention that the fenestrations in the intestine and endocrine glands represent true discontinuities in the blood vessels (72, 73).

However the capillaries in retina and central nervous system have an intact lining of endothelial cells and yet show no activity on or within the endothelial cells. If one postulated that in type I vessels with pinocytic vesicles the enzymes in the vesicles are concerned with transport across the endothelium, and in type III vessels with intracellular fenestrations no endothelial barrier

exists, one is still left with the problem of explaining the enzymatic basis of transport across the intact endothelium of type II vessels. Since the permeability properties of these type II vessels are alleged to be peculiar, however, one might expect that different enzymes are involved.

It is also conceivable that the enzymatic activity demonstrated in this study is not related to the transport of materials across the endothelim per se, but rather is concerned with special functions such as membrane formation in the case of the pinocytic vesicles or maintenance of electrolyte gradients across the glial and epithelial cell membranes.

Enzymatic Activity in Search of a Function

It is not surprising that a number of possible functions exist for the enzymatic activity demonstrated in this study. It is anticipated that further studies on the cytochemical properties of blood vessels will clarify and extend these observations and thereby provide someinsight into the chemical basis of vascular permeability.

Acknowledgement

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